An investigation in the neuromodulation of olfactory function using non-invasive vagus nerve stimulation

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Abstract

In the existing literature, invasive direct vagus nerve stimulation (VNS) using high frequencies has shown to be effective in modulating the activity of the olfactory bulb in animal models. Under indirect vagus nerve stimulation, stimulation via peripheral nerves (such as median nerve stimulation-MNS) using low frequencies alleviates nausea, which indicates a potential contribution of the olfactory system. However, there is no research that assessed the effects of median nerve stimulation (either high- or low frequency) or high frequencies VNS on olfactory function in humans. The present study aimed to, therefore test the potential effects of non-invasive, direct and indirect stimulation of the vagus nerve (VN), using high- and low frequencies in humans.

Two separate experiments were performed. In the first experiment, healthy adult male participants (n = 20) performed an odour threshold test (OTT), before and after receiving either, high-, low frequency MNS (indirect VNS) or placebo. Data from olfactory tests were analysed using paired parametric and non-parametric statistical tests (dependent on the presence or absence of normal distribution in the normality test using *Shapiro-Wilk* correction). Stimulation of the median nerve (MN) (indirect VNS) through high or low frequencies displayed no improvements in the performance of the OTT in healthy, adult male participants.

The second experiment explored the effects of non-invasive, direct VNS through the auricular branch of the vagus nerve (AbVN). Healthy, adult male participants (n = 18) performed an OTT and supra-threshold test (STT), before and after receiving either, high-, low frequency VNS or placebo. Supplementary exploration of bilateral orbitofrontal cortex (OFC) was performed using near-infrared spectroscopy (NIRS). NIRS data of separate stimulation parameters were statistically analysed using repeated-measures ANOVA across all stages of the experiment. Data from the olfactory tests were analysed using paired parametric and non-parametric statistical tests. Only direct auricular VNS under high frequency stimulation displayed improvements in olfactory function of the healthy participants in the STT (p:0.021, Wilcoxon sign-ranked test), with significant

differences in the NIRS recordings of the right hemispheric, OFC (p:0.014, post-hoc test with *Bonferroni correction*).

Overall, the results of the present study demonstrated that non-invasive auricular VNS using high frequency stimulation can improve STT performance in healthy humans for the first time in the existing literature. Findings from this study provide important insights into the potential effects of high frequency VNS over the olfactory function via the OFC. Further studies are needed to investigate the entire network of olfactory system that responds to high frequency VNS. In addition, the results of the present study also underline a potential beneficial effect of high frequency VNS on early progression of olfactory impairments in Alzheimer's and Parkinson's disease, which, in this context, future studies are also needed to investigate this potential.

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List of Abbreviations

Abbreviation	First Page Display	Full Extension
AbVN	Page i	Auricular branch of the Vagus Nerve
AD	Page 2	Alzheimer's Disease
ANZCTR	Page 28	Australian New Zealand Clinical Trials Registry
AOB	Page 4	Accessory Olfactory Bulb
AOS	Page 3	Accessory Olfactory System
DMV	Page 1	Dorsal Nucleus of Vagus
EA-	Page 19	Electroacupuncture
EC	Page 4	Entorhinal Cortex
EEG	Page 26	Electroencephalography
fMRI	Page 25	functional Magnetic Resonance Imaging
HbO_2	Page 26	Oxygen Haemoglobin
LC	Page 59	Locus Coeruleus
LN	Page 14	Lewy Neurites
MN	Page i	Median Nerve
MNS	Page i	Median Nerve Stimulation
MOB	Page 4	Main Olfactory Bulb
MOS	Page 2	Main Olfactory System
NIRS	Page i	Near-infrared Spectroscopy
NPvt	Page 59	Paraventricular thalamic nucleus
NTS	Page 1	Nucleus Tractus Solitarius
OB	Page 1	Olfactory Bulb
ODMT	Page 9	Odour Discrimination/Memory Test
ODT	Page 9	Odour Discrimination Test
OFC	Page i	Orbitofrontal Cortex
OIT	Page 9	Odour Identification Test
OM	Page 9	Odour Memory Test
ORT	Page 9	Odour Recognition Test

OT	Page 4	Olfactory Tubercle
OTT	Page i	Odour Threshold Test
PC	Page 4	Piriform Cortex
PC-6	Page 17	Pericardium acupuncture point-6
PD	Page 2	Parkinson's Disease
PET	Page 7	Positron Emission Tomography
Ppm	Page 37	parts per million
PVN	Page 58	Paraventricular Nucleus
rSO_2	Page 26	Regional haemoglobin oxygen saturation
rVLM	Page 61	rostral ventrolateral medulla
RN	Page 58	Raphe Nucleus
S-I	Page 17	Primary Somatosensory Cortex
S-II	Page 17	Secondary Somatosensory Cortex
SMA	Page 17	Supplementary Motor Area
STT	Page i	Supra-Threshold Test
TENS	Page 19	Transcutaneous Electrical Nerve Stimulation
UPSIT	Page 9	University of Pennsylvania Smell Identification Test
VN	Page i	Vagus Nerve
VNS	Page i	Vagus Nerve Stimulation
VNO	Page 4	Vomeronasal Organ
$VWrSO_2$	Page 34	Venous Weighted Regional Oxygen Saturation

1.0 Introduction [a]

1.1 General Introduction

In existing literature, use of direct VNS has demonstrated the ability to modulate the function of olfactory bulb (OB) in animal models (1). So far, the investigation of common nerves in their ability to alter the function of the olfactory system includes the olfactory, trigeminal, facial and glossopharyngeal nerves (2–8). However, no studies have yet tested non-invasive, direct or indirect stimulation of the VN as a potential neuromodulatory technique on olfactory functions. Neuromodulation of the VN was initially performed to treat epilepsy through an invasive, transcutaneous stimulation (9). Neuromodulation of the VN can follow a direct method, using invasive and non-invasive approaches, or an indirect method, using a non-invasive approach via the peripheral nerve stimulation (in the context of this thesis, MNS).

Previous studies using electrostimulation on the MN resulted in significant improvements on gastric mobility and alleviating symptoms of nausea and vomiting in both animal models (10–14) and human studies (13,15,16). This effect was understood to occur via VN brainstem nuclei including Nucleus Tractus Solitarius (NTS) and Dorsal Nucleus of Vagus (DMV) (14,17–19),. Previous literature has indicated that there is a role of gastric distension in the modulation of the OB in animal models (20–22). This could potentially underlie the effects of MNS on the activity of the OB (using VN neuronal network as an indirect pathway). Analogous to the indirect VNS techniques (through MNS), the use of direct VNS under high frequencies has also demonstrated an increase in the activity of the OB in animal studies (1). However, previous studies in human subjects have only explored the use of direct VNS under low frequencies as it fit the stimulation parameters for the conditions of the patients that were used in these studies (8,23).

[a] Work from this thesis has been published as a manuscript by Frontiers in Neuroscience (244).

Part of the main olfactory system (MOS), the OB play a key role in processing smell, in addition to several primary and secondary olfactory structures of this olfactory system in the cerebral cortex (24–28). Part of the secondary olfactory structures in the cortex, the OFC is considered the relay station of olfactory connections (29,30). The OFC has displayed increased activation in response to odour stimulus in neuroimaging techniques (NIRS) (31–33). Olfactory impairments are not only prevalent with ageing (34,35) but also in various neurodegenerative diseases such as Alzheimer's disease (AD) or Parkinson's disease (PD) (34,36–38). Both of the aforementioned neurodegenerative diseases present olfactory impairments from the earliest stages and therefore implores investigation into potential treatment options. In light of these potential benefits, this study focused on effects of different neuromodulatory techniques on olfactory function in the MOS.

The introductory chapters outline the relevant background information to the current thesis. First, a brief exploration on the anatomy and pathways of the olfactory networks is examined. The importance of olfactory function, alongside olfactory tests and subsequent olfactory dysfunction present in ageing and neurodegenerative diseases is detailed next. After the review on olfactory function, investigation of direct (auricular VNS) and indirect (through the stimulation of the MN) stimulation of the VN in current literature in addition to the neuromodulatory effects associated with these nerves is described. Finally, the aims and objectives of the thesis are provided in the final section.

1.2 Duality of the olfactory system anatomy

The role of the olfactory system is pivotal, in our interactions with the surrounding environment. This includes perceiving odours or detecting pheromones that play an important role in human social interactions (34,39,40). The olfactory system is our most primitive sense, yet is a sensory modality in which human beings exhibit plasticity throughout our entire lifetime. The mechanisms of neuronal plasticity in the olfactory system have been investigated at both cellular and cognitive levels (41–43). The importance of olfactory systems is highlighted through several neurodegenerative diseases that present early impairments in olfactory functions (mentioned in chapter 1.3.2 and 1.3.3) (34,36,38).

It is understood in the existing literature that vertebrate mammals use two distinctive olfactory systems: the MOS (*Figure* 1.1) and the accessory olfactory system (AOS) (44–47). Although evidence is scarce in the existing literature that these two systems present a dichotomy of olfactory function, it is understood that the AOS processes pheromones while the MOS is associated with processing general odorants (44–46). A previous hypothesis has been proposed that the AOS is responsible for processing large, high-weight molecules that are not volatile in the air. In comparison, the MOS processes low-weight molecules, which become airborne and contact the olfactory epithelium (47). The separation in function of the two olfactory systems could also be associated with their structural localisations. Vomeronasal sensory neurons (part of the AOS) are understood to be located in an enclosed tubular structure, where there is a restriction to the access of air-borne odorants. In comparison, the MOS sensory neurons are located on nasal turbinates' that are readily exposed to volatile odorants (44).

The AOS comprises of the vomeronasal organ (VNO) and the accessory olfactory bulb (AOB). VNO is located at the base of the nasal septum while the AOB is located at the dorsal-caudal surface of the main olfactory bulb (MOB). In these regions, the vomeronasal receptor axons connect with the centrifugal afferents from the nuclei of the AOS. The AOS helps control of neuroendocrine function and social behaviour (through processing of chemical compounds-pheromones). VNO, using bipolar neurons, project to the AOB, which processes vomeronasal input. Additionally, the AOB projects to the

amygdala (*Figure 1.2*), the bed nucleus of the stria terminalis and posteromedial cortical nucleus (44).

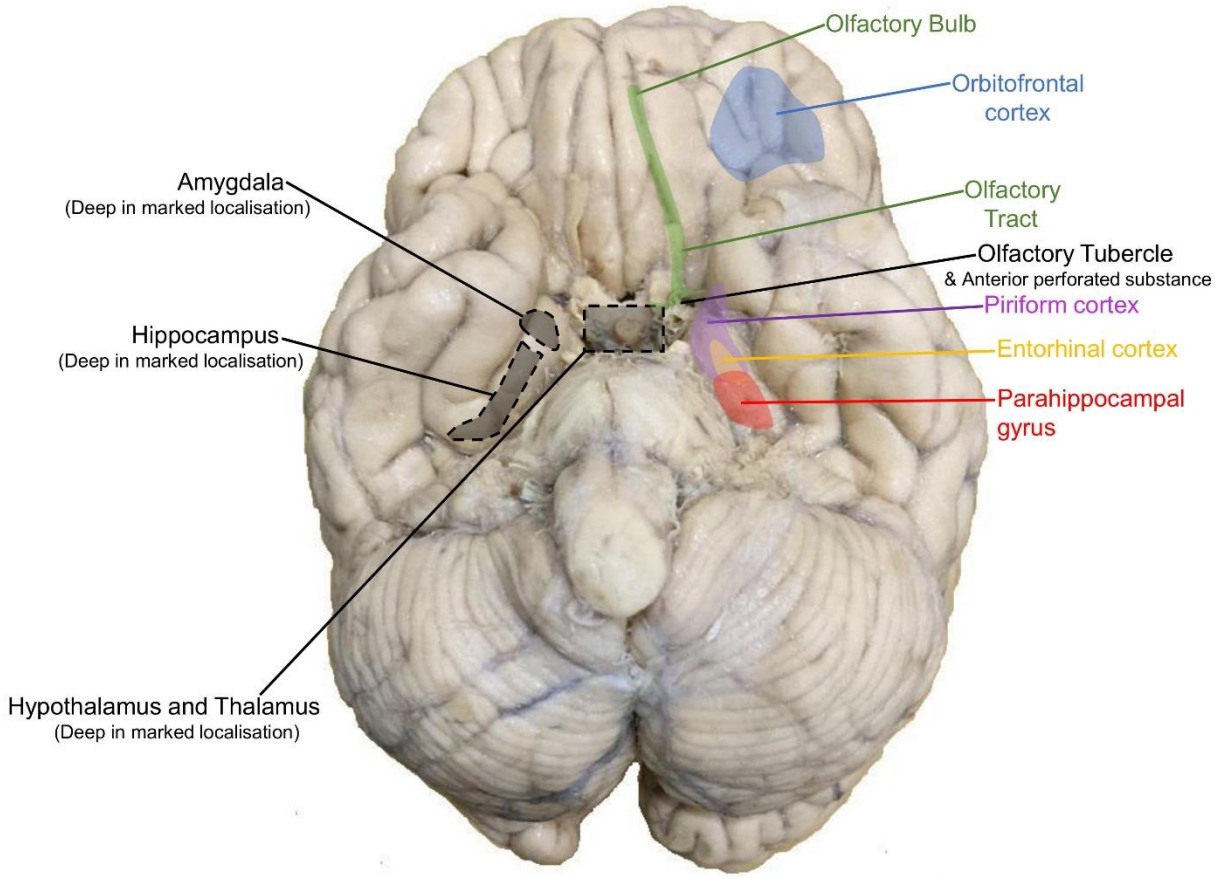

Figure 1.1 The location of primary and secondary olfactory areas of the main olfactory system in the human brain.
Labelled in the figure, primarily olfactory areas which include the piriform cortex (PC), entorhinal cortex (EC), parrahippocampal gyrus, olfactory tubercle (OT). The secondary olfactory areas that are labelled include the OFC, hippocampus, thalamus and hypothalamus. Image obtained and approved by the W.D. Trotter Anatomy Museum (Department of Anatomy, University of Otago, New Zealand), edited with labels of the primary and secondary olfactory areas.

The second olfactory system is the MOS. In the context of this thesis, discussions of the olfactory system will be in reference to the MOS. The MOS can be divided into primary olfactory areas [PC, OT, amygdala (periamgydaloid cortex) and EC] and secondary olfactory areas (OFC, hippocampus, hypothalamus and thalamus) (*Figures 1 and 2*)

(26,27,48). The PC and EC are two key regions of the primary olfactory areas of the MOS. The PC is interchangeably referred to as the primary olfactory cortex as it receives the majority of inputs from the OB. The PC is also the largest central olfactory areas, spanning the anatomical junction between the frontal and temporal lobe (referred to in two subdivisions-frontal piriform and temporal piriform cortex). The EC is considered the gateway for information leaving and entering the hippocampal formation and a key component of the medial temporal lobe memory system. This structure is one of the main terminals of the PC (34) and plays a large role in intrinsic memory functions such as maintaining stimulus-specific neural activity (working memory). This is suggested to be a crucial feedback pathway that is impaired (along with the PC) in neurodegenerative olfactory deficits (49).

1.2.1 Projection of odour signals to the primary and secondary olfactory regions of the cortex

Olfactory structures involved in processing odours are presented in *Figures* 1.1-1.2. In a short summary, perceived odorants reach the olfactory cleft and are passed through the mucus layer by the olfactory binding proteins. The olfactory epithelium is only located in the superior portion of the nasal cavity. Therefore, the majority of inspired air around and down the nasal pathways cannot reach the olfactory binding proteins. This could potentially work as a selective process where only odorants that are sniffed or drawn in, are processed through the olfactory system (39). These odorants bind onto the olfactory receptor neuron's cilia, each of, which have receptors that are specialised for a precise number of odours (50). Detailed in an article by Kovacs (26), peripheral bipolar receptor cells, numbering at millions per nose, are located in the olfactory epithelium of the nasal cavity. The axons of the olfactory neurons branch to the superficial layer (olfactory nerve layer) and synapse onto the glomerular layer of the OB.

The OB is the first communication structure responsible for processing smell. This structure hosts an intricate and complex internal circuitry, which is reviewed in an article by Arruda & colleagues (24). In summary, there are two types of excitatory/output neurons (mitral and tufted cells) and inhibitory interneurons

(periglomerular and granule cells). The cell bodies and dendrites of these neurons are organised in separate layers, which contributes to their unique olfactory functions (24). The olfactory nerve fibres connect to the mitral and tufted cells of the OB. It travels initially to the primary olfactory cortex (PC) and then, to the primary olfactory areas through the use of the olfactory tract and the OT (51,52). The olfactory information from the primary olfactory areas is then projected to the secondary olfactory areas (OFC, hippocampus, hypothalamus, thalamus) (26,27,48).

1.2.2 Orbitofrontal cortex, the 'processor-hub' of olfactory information in the cortex

The OFC is a crucial component of the MOS, operating as the main neocortical projection site for numerous olfactory regions in the cortex. This structure sends reciprocal feedback to the PC and EC, in addition to the primary and secondary olfactory areas (29,30). The OFC is located at the ventral surface of the prefrontal cortex and receives information from the taste, olfactory and somatosensory inputs (53). Although considered a part of the secondary olfactory cortex, this structure receives somatosensory inputs from the frontal and pericentral operculum, insular cortices, amygdala, mediodorsal nucleus of the thalamus and pars magnocellularis. The OFC also projects to regions of the temporal lobes that includes inferior temporal cortex, cingulate cortex, preoptic region, lateral hypothalamus (ventral tegmental area) and head of caudate nucleus (29,53).

The OFC is responsible for a diverse range of olfactory function. This structure is one of the primary areas in the cerebral cortex that represents smell (in addition to processing reward values of smell). The OFC is also associated with processing rapid reversal of behaviour by stimulus-reinforcement association re-learning), executive behaviours and represents positive affective aspects of somatosensory stimuli (27,29). Using positron emission tomography (PET), current literature has reported that perception, discrimination and recognition of odours all involve the orbitofrontal, cingulate and insular cortices (52). Separate functional imaging studies also reported an

increase in bilateral OFC activation (in comparison to the temporal, parietal or occipital lobes) under the presentation of odour stimuli (31–33).

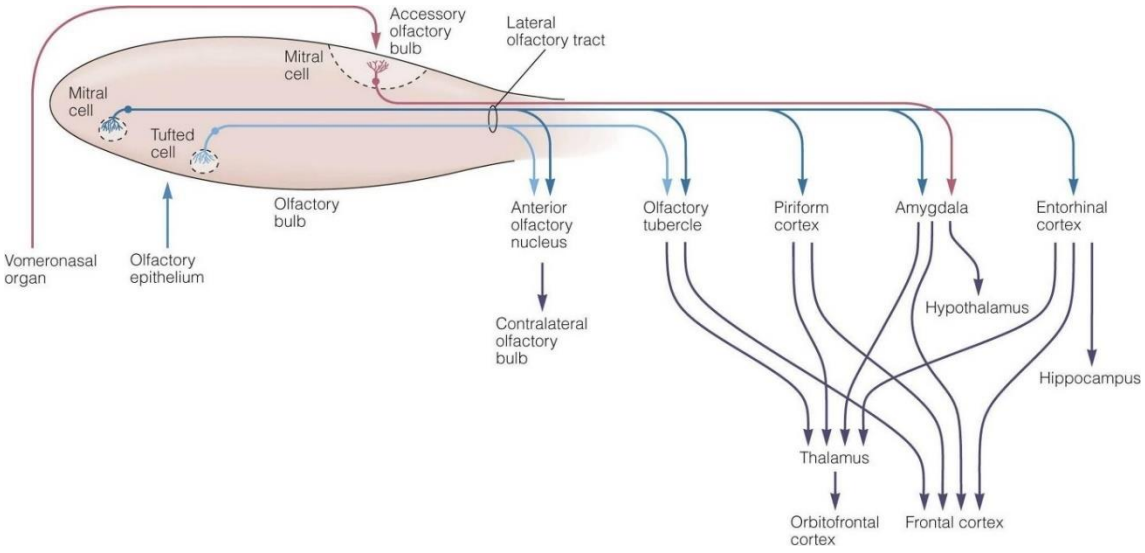

Figure 1.2. The pathway of the main and accessory olfactory system.
Pathways of the MOS are presented using blue arrows, with light-blue arrows for the primary olfactory areas, while the darker-blue/black arrows represented projections into the secondary olfactory areas of the cortex. The red arrows indicate the AOS. Retrieved from Kandel (54).

1.2.3 Functions of the olfactory system

Humans are thought to have a sense of smell that is inferior to other animals due to the decline in olfactory-gene numbers (55,56). However, this decline is compensated by a greater capacity to analyse complex smell signals due to the enlarged cortical centres (55,56). Even within the human population, there is a large variability in our ability to detect odours. There are some populations expressing the ability to detect several hundred odours while other populations express the capacity to detect several thousand odours (55).The first indication on how humans detect an odour came from Dr. L. Buck and Dr. R. Axel in 2004. They identified 350 different odorant receptors in the cell membranes of human olfactory receptor neurons and found that stimulating different patterns of olfactory receptor proteins (single or combination of specific odorant

receptors), were perceived as ‘unique’ odours in the brain (57). The olfactory receptors in the nose are very sensitive, capable of picking up faint odours. Our olfactory system also allows a rapid adaptation of olfactory sensations if there is prolonged presence of the same odorant. This is due to the inhibition of action potentials by specialised granule cells in the OB and by fatigue of odorant receptor function caused by ongoing, similar odour presentation (57).

Our ability to detect and perceive odours determines our preference to specific foods, which in turn, can have substantial impact on our health and our risk of developing chronic conditions such as obesity or diabetes (55). The olfactory system allows us to detect spoiled food or airborne toxins in our surrounding environment (34,39,55,58). The olfactory system can also have an influence in the kinematics of hand shaping (the reach to grasp movement). In an article by Bult & colleagues (59), the reach to grasp movement was indicated to be influenced by the presentation of odours. Specifically, it was indicated that the motor plan evoked by the odours were very fine-grained, to the extent that it could affect the pattern of angular excursion at the level of individual fingers’ joints and degrees of synergic movement amongst digits (59).

Our olfactory system can also contribute towards mating preferences. In a study by Wedekind & colleagues (60), a group of women were asked to rate the shirts worn by men (for two nights avoiding anything that could alter their ‘natural’ odour) under three criteria (intensity, pleasantness and sexiness). The results from the study indicated that women preferred T-shirts worn by men with different compatibility genes. This suggested that humans could potentially, in a subconscious manner, use smell to pick partners who present different compatibility genes that could be associated with a genetic advantage for the offspring (60).

1.2.4 Assessing olfactory function

There are two different types of olfactory processes when it comes to perception of odours. Orthonasal process, which occurs when odour molecules are delivered to the olfactory epithelium through the nares. The second is retronasal process, which occurs when odorant molecules from the oral cavity are delivered through the nasopharynx and posterior choanae to the olfactory epithelium in the olfactory cleft (61–63). Olfactory function can be assessed from different aspects with various tasks available. These include odour identification test (OIT- the subject is required to identify the name of a presented odour), odour discrimination test (ODT- a target odour is presented along with distinct odours and the subject must distinguish, which one is the target odour), odour memory test (OMT- the use of time delays between target odour presentation and the response of the subject for the target odour), OTT (the test of the minimal intensity that the subject can detect), odour recognition test (ORT- the test that requires the subject to recognise the name or substance of the presented odorant and STT (the test that assesses the subject's perception to odorants above threshold) (64,65).

There are several techniques available for investigating chemosensory function (e.g. several tasks mentioned in the previous paragraph). Suitable methods to access population-based olfactory assessment must involve a valid and sensitive measure that enables quantification of sensation and comparison across individuals. These methods should also provide a protocol that is a rapid, reliable, convenient, cost-effective and user-friendly. Currently, two techniques are commonly used for clinical studies.

University of Pennsylvania Smell Identification Test (UPSIT-OIT) and odour discrimination/memory tests (ODMT) provides a reliable measure, with a test-retest reliability above 0.90 (66). These tests also correlate more closely to clinical setting than traditional measures of olfaction (66). Previous literature has indicated that UPSIT tests enables the analysis reflective of central olfactory processing, but not of peripheral aspects of olfactory function (which can be measured through the OTT) (29). As an alternate method, the Sniffin' Sticks technique has been used frequently in past literature to observe measures of OTT (65,67–73). In the OTT, a measure of repeatedly ascending and descending concentrations of the same odorant is tested using the

Sniffin' Sticks technique. This enables the identification of the least detectable concentration for a particular odour (29). Quantitative olfactory testing methods such as UPSIT-OIT/ODMT or Sniffin' Sticks all involve orthonasal processing.

Using the Sniffin' Sticks method, an article by Hedner & colleagues (74) proposed the idea that cognitive variables for different types of olfactory tests could be associated with separate areas of the cortex. Specifically, they indicated that performance in the OTT could be driven by sections of the cortex that are responsible for low-order perceptual functions (volume of the OB related to the peripheral sensory input of olfactory processes). In comparison, performances in ODT, OIT, STT or tests of hedonic value of odours poses more cognitive demands and are represented in cortical areas that are responsible for higher-order olfactory function (74). Several studies have also concluded that the processes of OTT differentiate from other olfactory tests that require more cognitive processes including ODT, OIT, ORT, OMT and STT (70,73–76).

Initial methods of evaluating STT in odour perception began with the use of category rating scales. These methods were direct and allowed the measures of perceptual differences between subjects and in only a theoretical manner, explored the absolute strength of sensations. However, doubts were raised whether they could produce effective results across all sensory modalities (77) because the category scales are not spaced to give scale ratio properties (i.e. a rating of 6 on the scale is not twice as that of rating of 3). Instead, subjects were forced to assign sensations to categories that correspond to constant perceptual intervals with no true zero value, with the resulting data lying outside a ratio continuum (78–80).

An alternative method to access STT is to use varying odour intensities of the same odour and observe if participants can identify the target odours (81). This methodology avoids the issues associated with the use of category rating scales mentioned previously. It is understood in the existing literature that STT functions are an important element of the olfactory system and STT results do not show correspondence with other olfactory measures, especially the OTT (82,83). Therefore, this thesis examined olfactory

performance with both OTT-representing low-order olfactory function and STT-representing higher-order olfactory function (74).

1.3 Olfactory dysfunction in ageing and neurodegenerative diseases

1.3.1 Ageing

About half of the elderly population between the ages of 65 to 80 years present olfactory impairments (64,84,85). Olfactory impairments are present in about 3.8% of adults between 21-84 years of age. The presence of olfactory impairment increases with age from 0.6% under 35 years of age to 13.9% at 65 years of age or older (86). Age is suggested to exhibit a negative influence across all olfactory tasks (64,68,74,87), alongside sex where female participants outperform the male counterparts in olfaction sensitivity tests. The prevalence of olfactory impairments is also higher in men than in women (74,88). The loss of olfaction with ageing has been linked to changes in non-olfactory elements of the nose (airway patterns or mucous composition), olfactory neuroepithelium (reduced surface area), OB function (reduction of mitral cells in the OB) (26,89,90), central brain regions involved in olfactory functions and neurochemical changes in the brain (36,91).

1.3.2 Alzheimer's disease

AD is characterised by neuropathological changes, which includes neurofibrillary tangle formation, neurotic plaques and atrophy. This progressively leads to deficits in functions of memory (such as amnestic presentation), language and visuospatial capacities (36,92). The presence of olfactory impairments at the early stages of AD has been linked to several factors occurring initially at the EC, one of the main terminals for the PC and neuropathological changes in the OB (35,36,93,94). Nobel prize winners M.

& E.B. Moser also demonstrated in 2005 that the nerve cells in the EC represent ‘grid cells’ that helps with spatial navigation alongside ‘place cells’ in the hippocampus. This was discovered in 1971 by Nobel prize winner, J. O’Keefe (95), who indicated that EC acts as a personal human guidance system (96). This raises the question whether deficits in EC that lead to impairments in spatial navigation in patients with AD could, in fact, be a result of olfactory impairments present in the EC, one of the primary olfactory cortical areas in the human brain.

In all patients with AD, mitral cells in the OB present AD-type pathology (26,97). This includes the presence of excess tau and amyloid precursor protein (morphology of the molecular compounds of AD) (98–100), in conjunction with reduction of cerebral blood volume (101) and thinning of the EC (main end-terminal for the olfactory system) (102,103). AD presents a characteristic pattern of neurofibrillary tangle formation that initiates in the entorhinal region and the adjoining temporal neocortex. This pattern of AD-pathology then extends to other structures in an expected manner (detailed in *Figure* 1.*3*) (104). According to an article by Braak & Braak (104), the sequence of neurofibrillary tangle formation in patients with AD allows the distinction of three main stages: transentorhinal, limbic and neocortical stages. Impairments in the EC are also perceived as the link to olfactory dysfunction in the associated interconnected cortical regions (101).

Looking at the performance of olfactory tests in patients with AD, ODT (105), ORT (69,106–108) and OIT (69,70,72,107,109–113), are impaired from the earliest stage of AD. Olfactory dysfunction in the aforementioned testing modalities in patients with AD also represents olfactory functions that require associated cognitive load. In comparison, impairments in OTT in patients with AD are present in later stages of the disease in contrast to early impairments present in OIT, ODT or ORT (70,72,75,108,109,111,114–116).

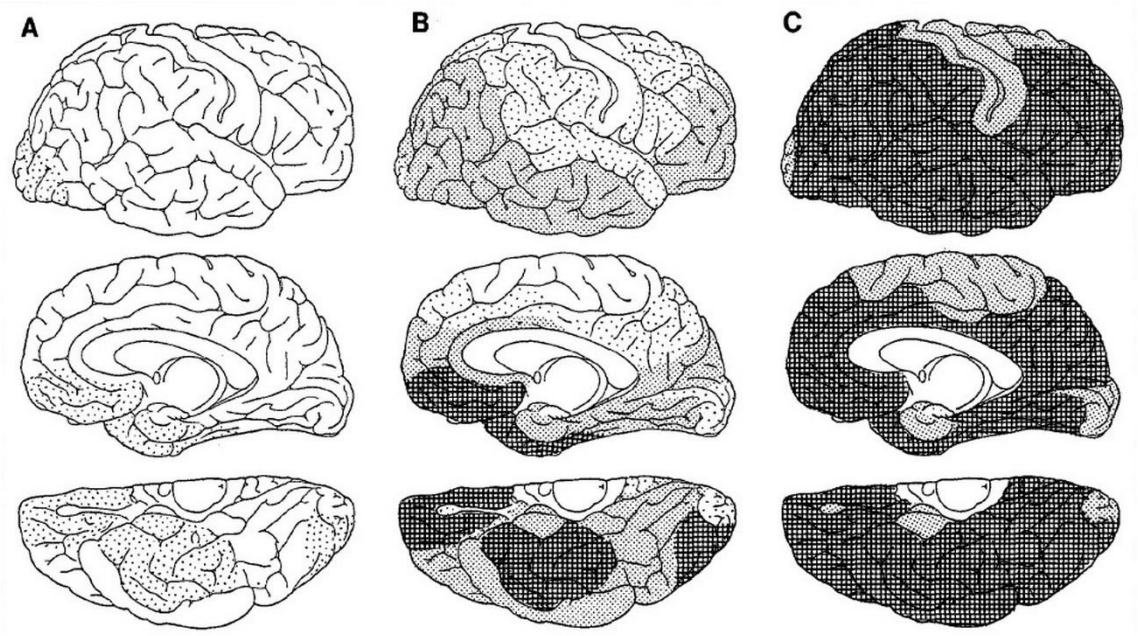

Figure 1.3. Distribution patterns of AD pathology (amyloid deposits).
Stage 1(**A**) shows AD pathology in basal sections of the neocortex. Stage 2 (**B**) shows AD pathology in all neocortical association areas. Stage 3 (**C**) shows AD pathology in all areas of neocortex including sensory and motor association areas (darker regions suggest increased number of AD pathology accumulation). Retrieved from Braak & Braak (Pg. 244; original publisher: BioMed Central) (104).

1.3.3 Parkinson's Disease

PD is associated with a reduction of nigrostriatal dopaminergic neurons in distinctive circuits of the brain (36,117), with impairments including tremor, bradykinesia of movement and postural instability. Due to these impairments, patients with PD often present poor balance and coordination in movement (36). Olfactory impairments in patients with PD are also one of the earliest non-motor symptoms, preceding motor symptoms by several years (34,118). These olfactory impairments can differentiate patients with PD from healthy controls better than the clinical motor tests (34,119), single-photon emission computed tomography imaging of dopamine transporters (34,120) or patients with neurological conditions that resemble PD. This includes neurological conditions such as progressive supranuclear palsy (121,122), multiple system atrophy (122,123), corticobasal degeneration (122) or essential tremor

(124,125). In patients with PD, impairments were present in OIT (69,124,126,127), ODT/ORT (69,73,106) and STT (91) from the earliest stages of the disease.

Impairments in patients with PD progresses in neuropathological stages, which are marked by continuous development of distinctive inclusion body (branching Lewy neurites- LN) within cellular processes and as granular aggregations and spherical pale bodies (128,129). LN are present from the earliest stage of PD, identified in the anterior olfactory nucleus, the OB, and the DMV (130). The spread of LN throughout the stages of PD (*Figure 1.4*), initially begins at the DMV. PD-pathology is initiated here due to due to the presence of large-long projection, unmyelinated preganglionic fibres (which are more vulnerable to intraneuronal aggregates associated with PD pathology). These preganglionic fibres connect the central nervous system with the postganglionic nerve cells of the enteric nervous system (128,131,132). This pathology spreads throughout the subcortical regions and eventually reaches the cortical areas in later stages of PD (34,128,133). The spread of PD pathology indicates the existence of a connection between the VN and the olfactory processing areas of the cortex that facilitates the origin of early olfactory dysfunction in PD (128,133,134).

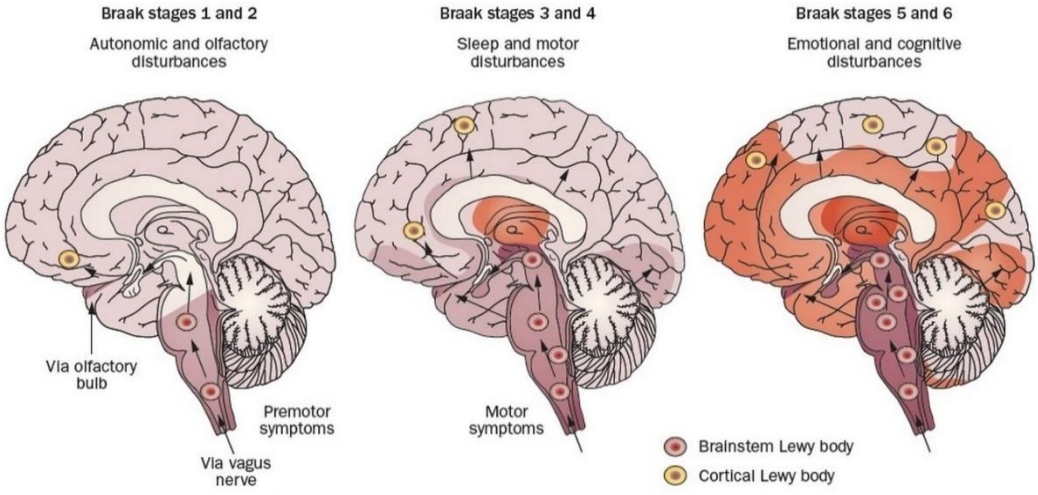

Figure 1.4. The Braak stages of PD pathology.
In this figure, initiation sites for the pathology of PD can be observed from the OB and medulla oblongata, which then progresses into cortical regions. The diagram suggests that α-synuclein-related pathology is initiated in the periphery from the olfactory epithelium or vagal inputs and eventually leads to widespread α-synuclein-related pathology across the cortex (Braak stages 5 and 6). Retrieved with permission from Doty (34) (Licence no: 4335121200258).

1.4 Stimulation techniques of the vagus nerve and background of olfactory system modulation studies

Neuromodulation of the VN can be performed using a direct or an indirect approach. Direct stimulation of the VN can be invasive or non-invasive. Direct invasive VNS can be performed using an implantation of stimulator devices over the cervical segment of the VN with surgery (9,135). An example of this method includes the 'NeuroCybernetic Prosthesis' system (size of a pocket watch), which is implanted into the chest wall (left side) with bipolar electrodes that are wrapped around the left VN [*Figure 3* in George & Colleagues (136)]. Direct, non-invasive VNS procedures comprise of transcutaneous stimulation of the cervical segment of the VN [*Figure 3* in Yuan & Silberstein (9)] or the auricular branch of the VN (*Figures 1.6 and 2.4 A-B*) (9,137). In comparison, indirect, non-invasive VNS includes the stimulation of peripheral nerves (*Figure 2.2*), which can antidromically (conducting nerve impulses in the opposite direction in comparison to the standard) stimulate VN brainstem nucleus. So far, the stimulation of common peroneal nerve and MN results in the activation of the VN brainstem nucleus (such as the NTS) (138). So far, there is no invasive, indirect stimulation studies of the VN in the existing literature.

In the current literature, effects of neuromodulation on several cranial nerves and its subsequent effects on olfactory functions have been investigated. This includes the olfactory, trigeminal, facial, glossopharyngeal and vagus nerves(2–8). As chemical sense shares a mutual interaction (e.g. in aroma perception during eating, a combination of gustatory, olfactory and trigeminal information is processed), studies into cranial nerve interactions with olfactory functions have been studied collectively. Some studies have addressed specifically the trigeminal nerve as it has been understood to play a large role in perception of single odorant or mixtures of odours (2,4,6,7). However, relatively little investigation has been performed in the existing literature into the interaction between the VN, using direct or indirect (MNS) VNS on olfactory function (8,139). In 1984, using direct, invasive cervical VNS, Garcia-Diaz & colleagues have demonstrated an increase in the activity of the OB after high frequency (80 Hz) VNS, but not after low frequency VNS (10 Hz) in animal models (1). Frequency is defined as the number of occurrences of a repeating event per unit of time. In this respect, low frequency stimulation incurs more time between repeated events in comparison to high

frequency stimulation. So far, VNS in human subjects have used invasive, approaches under low frequencies, which could not achieve the same improvement in olfactory function (8,23). There are currently no studies exploring the use of direct or indirect methods of VNS under high frequency stimulation for a potential effect on olfactory function in humans.

1.4.1 Use of indirect, non-invasive vagus nerve modulation through the median nerve

1.4.1.1 Anatomy and functions of the median nerve

The MN is a branch of the medial and lateral roots of the brachial plexus. The MN runs from the axilla into the arm and passes through to the forearm (between the humeral and ulnar heads of the pronator teres) where it innervates the flexor muscles (with the exception of flexor carpi ulnaris - *Figure 1.5*). The MN passes through the midline of the wrist and after passing through the carpal tunnel to the hand, innervates the thenar muscles and lateral two lumbricals in the hand. The MN innervates the thumb, index finger and the middle finger (140). Specific innervation of the MN on muscles of the forearm and the hand are indicated in *Figure 1.5.* The primary motor and sensory function of the MN is represented in the primary and secondary somatosensory cortex (S-I and S-II respectively) (141–144) alongside several accessory areas of the cortex (such as supplementary motor area; SMA) (140).

1.4.1.2 Median nerve stimulation in the existing literature

Stimulation on the MN was initially performed using manual acupuncture. This was an ancient, traditional Chinese method used on the Neiguan acupuncture point (pericardium meridian of acupuncture point 6; PC-6) on the MN. Using this method, MNS was performed for treating various functional diseases. This included gastrointestinal diseases (145,146), gastroparesis (147,148), functional dyspepsia (15,149), constipation (150) and irritable bowel syndrome (151–153). Alternatively,

stimulation of the MN has also been implemented in alleviating symptoms of nausea or vomiting in clinical settings (such as post-surgery or chemotherapy, pregnancy and motion sickness). In addition, it is also used for other clinical conditions such as dealing with addiction, reducing stroke, reduction of headache, menstrual cramps and tennis elbow (11,12,15,16,142,143,154–156).

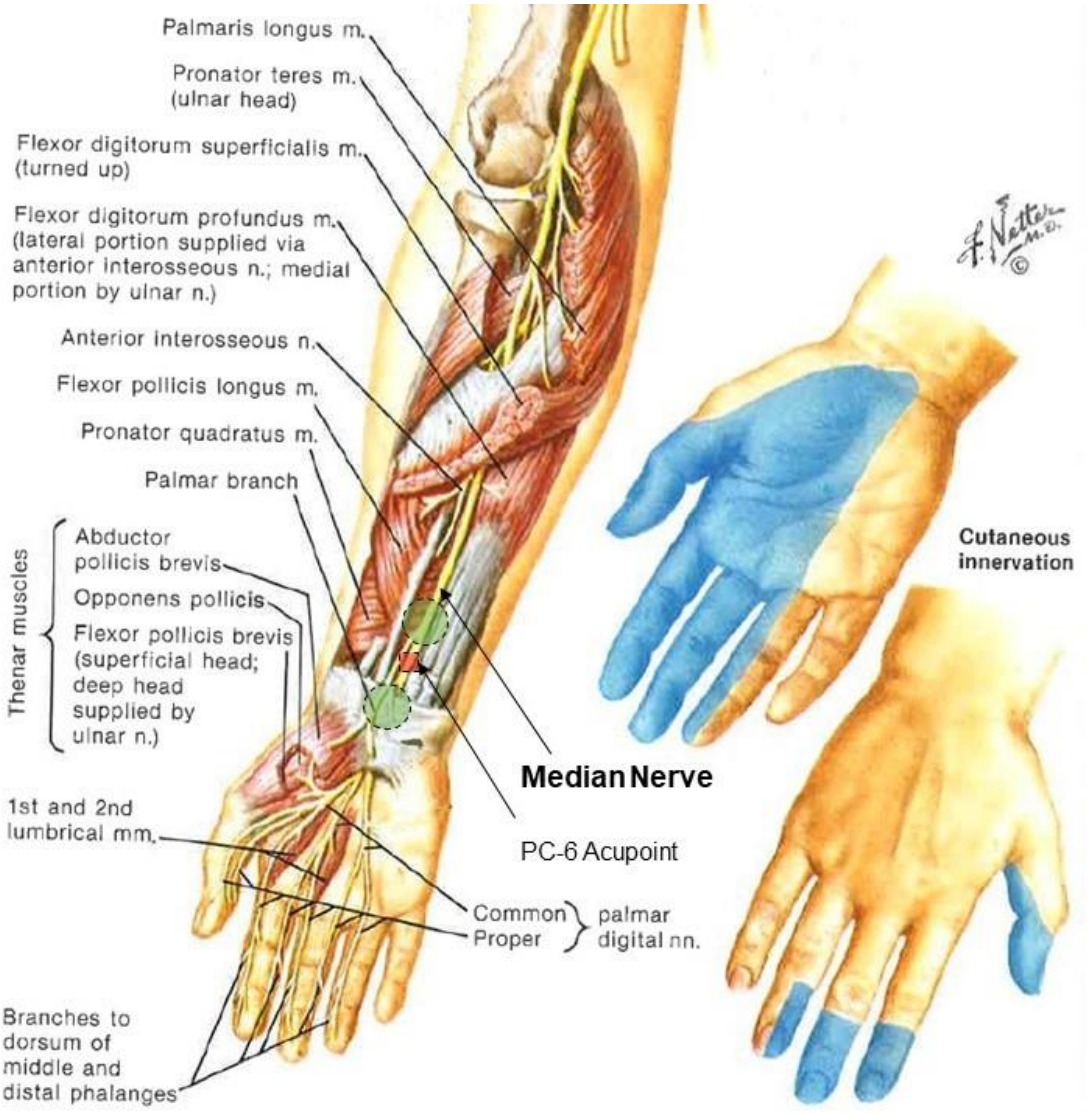

Figure 1.5. Displays the trajectory of the MN through the forearm and hand. All flexor muscles in the forearm (exception of flexor carpi ulnaris) that is innervated by the MN is included along with the thenar muscles of the hand. Blue regions highlighted in the images of the hand (right-hand side) indicate the innervation of the MN on the digits of the hand. Additions to the original image includes the label of MN and PC-6 Acupoint (Neiguan acupuncture point; red square zone) using black arrow heads and electrode stimulation sites (labelled using green zones) in *Experiment 1*. Retrieved and edited from Netter & Colleagues (140).

1.4.1.3 Use of median nerve stimulation (indirect vagus nerve stimulation) on gastric mobility and alleviation of nausea and vomiting

Over the past couple of decades, MNS progressed from manual acupuncture techniques (10,13,142) to electroacupuncture (EA-a modification of the acupuncture technique using electrical current) (10,12,14,144) and more recently, the use of transcutaneous electrical nerve stimulation (TENS-detailed in chapter 2.1.3) (10,157–159). The use of low frequency (10-25 Hz) EA-MNS has demonstrated an effective control of gastric mobility through increased gastric emptying and the ability to normalise gastric dysthymia (12,14,15). The VN is understood to be solely responsible for the control of gastric acid secretion, which is also modulated indirectly through the use of MNS in animal (14,17) and human studies (15,16). In this context, the MN can be understood to stimulate the VN in an indirect manner. This demonstrates the potential effects of MNS on olfactory regions through an indirect stimulation of the VN.

In a previous article by Ouyang & colleagues (17), low frequency MNS accelerated gastric emptying of liquid and improved gastric slow-wave rhythmicity. Both of these functions were attributed to an indirect stimulation of the VN using MNS. Furthermore, effects on gastric function using MNS did not occur after vagotomy, similar to other studies using animal models (15,19). In a similar manner to improving gastric function, past literature has indicated that MNS can be used to ameliorate nausea and vomiting through the VN (10–16,155,160). Furthermore, after bilateral truncal vagotomy was performed in animal models, alleviation of nausea and vomiting via MNS were substantially reduced (14,161). Sense of smell, along with senses of vision and taste, are key contributors to inducing nausea or vomiting as the first line of defence against dangerous or contaminated food (162). This suggests the potential role of MNS on olfactory function.

It is indicated that the effects on gastric function (increased gastric motility) through MNS occurs through the indirect stimulation of the VN (14,15,17–19). The potential pathway could be through the NTS where the MN projects to the DMV, which enables the modulation of gastric function (21,138,163). Both the DMV and NTS are VN brainstem nuclei. It is also indicated in past literature that VNS is proportionate to

gastric distension (20,164,165). In addition, the periglomerular cells of the OB, which respond to gastric distension also respond to VNS in a similar manner (20–22). Therefore, it could be hypothesised that the effects of MNS could potentially follow the same trajectory of the VN, through the NTS to areas in the cortex (166,167) that correspond to processing olfaction (with connections to the periglomerular cells in the OB). Although these previous studies support the indirect stimulation of the VN (through MNS), there are no neuronal tracing studies looking at the interconnection between the neural pathways of the MN and the VN brainstem nucleus. However, there are studies that indicate that the MN can activate the NTS (19,138,156,163,168,169). Although existing literature on MNS has investigated the effects of MNS on the S-I using low frequencies (141–144,154), to date, there are no studies looking at the effects of MNS, with either, high or low frequencies, on its potential effects on cortical regions of olfactory functions or olfactory function.

1.4.2 Use of direct, non-invasive auricular stimulation of the vagus nerve

1.4.2.1 Anatomy and function of the vagus nerve

The VN is the tenth cranial nerve, with the superior segments attached to the medulla through multiple rootlets. The VN exits the skull through the jugular foramen and travels down the neck as the cervical segment of the VN. Each of the cervical segments of the VN lies within the carotid sheath, between the carotid artery and the jugular vein. The VN contains sensory and motor fibres and is considered the ‘wanderer’ as it has a large range of distributed branches throughout the whole body (57). Dorsal to the skull, the VN leaves the jugular foramen to pass through the facial canal and distributes to the posterior aspect of the external ear canal and external ear (18,137,170). Sensory fibres of the VN innervate a number of structures that includes the larynx, trachea, heart, lungs, oesophagus, stomach, small intestine and gallbladder. The cell bodies of these sensory VN fibres lie in the jugular and nodose ganglia and terminate in the medulla. The somatic motor fibres of the VN innervate the muscles responsible for swallowing, with most of the motor fibres being autonomic (parasympathetic fibres). They are

thought to originate in the medulla and extend to various areas of the autonomic ganglia and finally the muscles of the pharynx, larynx, thoracic and abdominal organs (9,57).

The VN consists of three afferents (sensory; general somatic afferent, general visceral afferent and special visceral afferent) and two efferent types (general visceral efferent and special visceral efferent). They project to four different vagus nuclei: spinal nucleus of the trigeminal nerve, NTS, DMV and nucleus ambiguous. In the context of this thesis, we will focus on the NTS and DMV brainstem nucleus of the VN. The NTS is a series of nuclei, organised in a topological manner and handles the major viscerosensory information of the VN. In the vago-vagal reflex, this structure receives signals (mechanical and chemical stimuli) from the gut wall receptors. In response, the NTS integrates hormonal and visceral neural signals and sends feedback to the DMV. In contrast, the DMV is the major motor brainstem nuclei of the VN and transmits monosynaptic vago-vagal feedback to the gastrointestinal tract and plays a key role in gastric motility and secretion (21,135).

Looking exclusively at the sensory auricular VN (Arnold branch), it can be stimulated through specific portions of the external ear. The ear can be separated into multiple sections (*Figure 1.6A*). The external portion of the ear consists of the auricular and the external auditory meatus (ear canal), closed off from the inner ear by the tympanic membrane. Specifically, the concha, is separated by the crus of the helix into an upper (cymba) and lower (cavum) parts. In previous studies looking at the AbVN, it is indicated that the AbVN reach the external ear in humans with the aid of two divisions that are formed in the facial canal. One division follows the posterior wall of the external ear canal to the cavum and cymba conchae area of the auricular skin (*Figure 1.6B*) while the other division moves through the posterior auricular nerve of the facial nerve, following the route of the posterior auricular nerve in the posterior aspect of the external ear (*Figure 1.6C*) (171–173).

The AbVN (Arnold branch) is only distributed to the helics minor muscle zone located at the cymba concha region of the external ear (*Figure 1.6B*) (173,174). The majority of the medial and lateral surfaces of the external ear is innervated by the great auricular

nerve (third cervical nerve). The medial surfaces are innervated by the AbVN while the superior surfaces are innervated by the lesser occipital nerve. The lateral external ear is also innervated (to a lesser extent of the third cervical nerve) by the AbVN and in the superior regions, the trigeminal nerve. There are also some minor innervations through the glossopharyngeal nerve and the first and second spinal nerves (174–176).

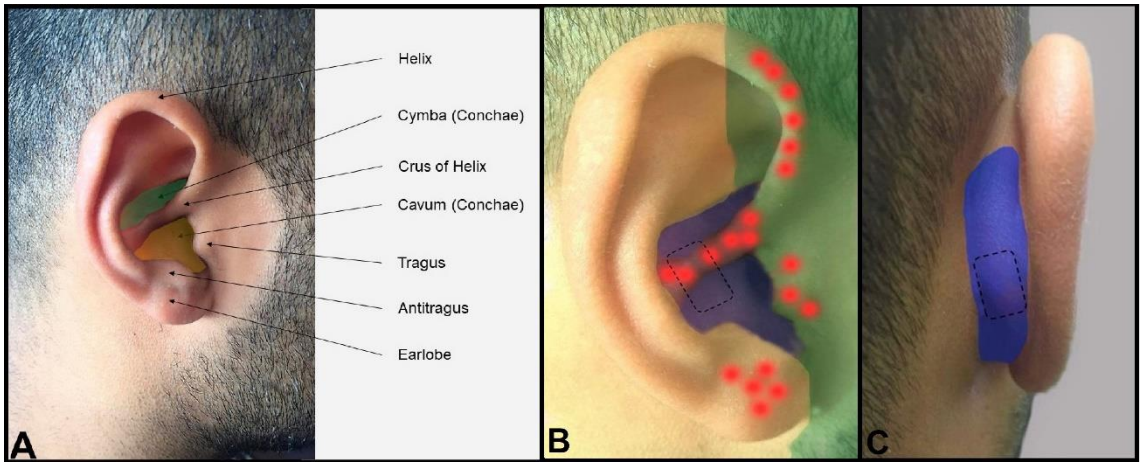

Figure 1.6. Anatomy of the external ear, including innervation zones of cranial and spinal nerves and electrode placement regions of *Experiment 2*.
A) Anatomical divisions of the external ear. The concha region of the external ear is divided into two divisions (superior portion pertaining to cymba conchae-green region while inferior portion is the cavum conchae-yellow region). The figure is modelled from Alvord & Farmer (174). **B)** Anatomical picture of the ear highlighting the area of innervation of the AbVN (blue regions over cymba and cavum conchae), facial nerve (innervation represented using circular red sites), trigeminal nerve innervation (green region), C2 spinal nerve innervation (yellow region). The figure is modelled from Cakmak & colleagues (177). Electrode placement in *Experiment 2* marked with black dotted line. **C)** Posterior aspect of the external ear, with the auricular VN innervation marked in blue region. The figure is modelled from Peuker & Filler (170). Electrode placement in the posterior aspect of the external ear is marked with black dotted line.

In animal models, tract-tracing method using an application of horseradish peroxidase was used to show that the AbVN projects to the NTS, spinal trigeminal nucleus and other sensory nuclei in the brainstem (178). This was also consistent with studies in human subjects (137,178–180). In humans, auricular VNS resulted with activation of

primary and higher-order central projections of the VN (such as the insula, amygdala, nucleus accumbens, hypothalamus and brainstem) (178–180). Auricular VNS can also stimulate the NTS (137) when the stimulation was provided to the conchae region of the external ear. In addition, stimulation of the AbVN in human subjects performed outside the conchae region of the external ear reported no evidence of NTS activation (179–181). Therefore, in this context, the use of auricular VNS displayed activation of the VN brainstem nuclei (NTS).

1.4.2.2 Background of vagus nerve stimulation and its interactions with olfactory bulb

The first use of VNS was initially performed by J. Corning in the late 19th century to treat epilepsy (9,182). Until the mid-1980s, it was not understood if there were opportunities to use electrical stimulation of the VN as a means of potential therapy (9). This changed in 1992 when the use of VNS showed the ability to stop seizures in animal models (183). This led to interest in the field of neuromodulation, using VNS in human studies. It was commonly performed in the left cervical VN as a potential therapy for patients with intractable/medically refractory epilepsy (9,138,184,185). Currently, invasive VNS is currently available for multiple conditions that include intractable epilepsy, chronic treatment-resistant depression (186), pain (186,187), primary headaches and medication-overuse headaches (188).

In a previous article by Garcia-Diaz & colleagues (1), invasive VNS under high frequencies demonstrated the ability to modulate the neuronal activity of the OB for the first time, on animal models. This study indicated that the ipsilateral OB neurons displayed an increase in firing activity under high frequency (80 Hz) VNS but not under low frequency (20-40 Hz) VNS in rat-animal models. In subsequent studies, invasive VNS in human patients with medically intractable epilepsy (8) and therapy-resistant depression (23), used low frequency stimulation, where no significant effects of VNS on functional olfactory tests were found. However, the use of high frequency VNS, previously demonstrated in animal models to be effective in modulating olfactory performance (1), was not performed. In fact, present research in VNS still needs to

address whether high frequency VNS, as demonstrated in previous research on animal models (1), is capable of modulating olfactory performance in human subjects.

1.4.2.3 Exploration in the beneficial use of non-invasive, direct vagus nerve stimulation

In one example of invasive neuromodulatory technique directly to the VN (Neuro Cybernetic Prosthesis -VNS), electrodes are attached to the left side VN below where the superior and inferior cervical branches come off. The use of unilateral left side VNS has shown bilateral functional projections into the brain (8,189) while reducing its innervation on cardiac function in comparison to the right side VN (134,190). However, with the use of invasive VNS, several issues needed to be addressed due to the invasive nature of neuromodulation. This includes cases of delayed arrhythmias or syncope after long-term use (191), VN trauma causing unilateral vocal cord dysfunction and dyspnea (192), sleep apnea (193), paraesthesia and pain (194). Majority of invasive VNS studies exhibit small patient samples, due to the difficulty in obtaining ethical approval to use this method on healthy subjects (9,137).

Previous studies in animal models have demonstrated that the auricular VN can activate the NTS (178,195). This was followed up in a recent article by Frangos & colleagues (137) exploring the use of non-invasive auricular VNS through the cymba conchae in human subjects. In this study, the use of electrostimulation at the conchae region of the external ear had a significant effect on the central projections of the VN, including the NTS and multiple areas of the cortex (subthalamic nucleus, periaqueductal gray, thalamus, amgydala, insula, nucleus accumbens, hypothalamus, bed nucleus of the stria terminalis and additional higher order projections) (137). This was the same neural circuits activated by invasive, direct VNS in the existing literature (196,197). Therefore, this highlights the feasibility to use non-invasive approaches of VNS that avoids invasive issues associated with using invasive VNS.

1.5 Exploration of the olfactory network using near-infrared spectroscopy

Neuroimaging as a technique provides an opportunity to detect activation of the cortex in association with olfactory tests and different neuromodulatory techniques. Neuroimaging techniques such as functional magnetic resonance imaging (fMRI) and NIRS allows for non-invasive measurement of cerebral activation, along with high spatiotemporal resolution and detection of metabolic changes that could be potential precursors to diseases. These techniques also allow the monitoring of the brain during or in response to treatment and the data from neuroimaging techniques also allows for quantitative analyses (198–201). In the context of this thesis, the focus will be on the NIRS.

1.5.1 History of near-infrared spectroscopy

The first use of spectroscopic measurement was used on *in vivo* tissue in 1932. In this study, optical characteristics of haemoglobin was observed (202). This study was followed up ten years later with the use of a practical ear oximeter for aviation purposes (203). This technique was used in a study by Millikan (203), which modified the previous technique to obtain absolute values of oxygen saturation of arterial blood (204). This was used till the 1970s as a clinical method of ear oximetry. There were still limitations regarding the stability of measuring continuous monitoring of oxygen saturation in the presence of superfluous assumptions for the calibration process. These issues were addressed in 1974, with the use of pulse oximetry. Pulse oximetry utilised the pulsation of arteries, allowing for an accurate measurement of oxygen saturation of arterial blood directly. Using this method, several studies in the 1980s demonstrated the effectiveness of the NIRS as an useful, non-invasive technique for rapid detection of tissue oxygenation (205–208).

In past literature, electroencephalography (EEG) has been used to detect changes in brain activity under different odour patterns (209–211). This type of technology, however runs the risk of data misinterpretation due to spatial smearing that occurs during the recording of the scalp EEG, in addition to presenting data with low

spatiotemporal resolution (31). Conversely, numerous studies have displayed the effectiveness of the NIRS to eliminate the issues of EEG and present high spatiotemporal resolution (31–33). NIRS uses the modified Beer-Lambert law for the light attenuation changes through the illuminated tissue, allowing a non-invasive measure of tissue oxygenation (199). The use of regional haemoglobin oxygen saturation (rSO_2) measurement in the NIRS allows the recording of continuous and non-invasive measure of cerebral regional oxygen balance (oxygenated and deoxygenated haemoglobin concentration) in the cortex (31–33,212,213).

1.5.2 Use of near-infrared spectroscopy in conjunction with olfactory testing

In past literature, several studies has used NIRS to monitor the activation of the OFC during the presentation of olfactory stimuli (31–33,212–214). Based on previous literature on fMRI techniques, an article by Harada & colleagues (31) observed the effects of smelling different odours (vanilla essence, strawberry essence or 3-methylindole) on the OFC, temporal lobes, parietal lobes and the occipital lobes using NIRS. They found that oxygen haemoglobin (HbO_2) concentration only increased over the OFC during the presentation of olfactory stimulus (31). This was supported by research that followed, using NIRS where increased OFC activation was present after the presentation of olfactory stimuli (33). NIRS results in the previous studies (31–33) are also supported by fMRI studies where olfactory impression is indicated to be processed in the lateral and anterior orbitofrontal gyri of the frontal lobe (157,215–221). Specifically in primate models, neurons in the OFC only displayed responses to the presence of olfactory stimulus (217). All aforementioned studies support the use of NIRS in measuring cortical activation of the OFC under olfactory testing modalities.

1.6 Present aims and objectives

In summary, existing literature indicates the potential of direct (auricular VNS) and indirect (MNS) stimulation of the VN in the modulation of olfactory function. The use of MNS in past literature in both animal and human studies has demonstrated a positive effect in gastric mobility (14,15,17,20–22) and alleviation of nausea and vomiting (10–16,155,160). Both of these effects using MNS occurs indirectly through the VN. Separately, the use of direct, high frequency VNS has shown to be effective in directly modulating the activity of the OB in animal models (1). Stimulation of the MN is also understood to improve gastric mobility through the DMV and NTS (VN brainstem nucleus) (21,138,163). If vagotomy is then performed, no effects of MNS were observed on gastric mobility (15,19) or nausea/vomiting (14,161). Although direct VNS under low frequencies have been investigated on the potential effects in olfactory function using invasive measures in human models (8,23), the use of non-invasive, direct or indirect, high frequency VNS has not been explored in the existing literature.

Therefore, to address the aforementioned gaps in the existing literature, the present study was conducted in two separate parts. In part one, the effects of non-invasive, indirect VNS (using MNS) on olfactory function was investigated, using high- and low frequencies, in healthy, adult, male participants. Olfactory function was assessed using OTT, before and after each of the stimulation parameters (high-, low frequency MNS and placebo). In part two, effects on both lower-level olfactory function (OTT) and higher-level olfactory function (STT) (74,76) were investigated after non-invasive, direct auricular VNS (using high- and low frequency stimulation and placebo) in healthy, adult, male participants. Supplementary NIRS recording of the OFC was also included in the second experiment to observe the activation of the OFC during olfactory testing modalities and neuromodulation of the VN.

2.0 Methods

2.1 Experiment 1: Use of median nerve stimulation (indirect vagus nerve stimulation) to modulate olfactory function

2.1.1 Participants

Twenty Caucasian-male, healthy, non-smokers (age range = 22-28 years, mean: 24.8 years, standard deviation: 1.75 years) participated in *Experiment 1*. Prior to the experiment, each of the participants were asked not to consume any food or beverages (aside from water) for two hours prior to the experiment. They were also instructed to refrain from applying any fragrance product/s on the day, prior to the experiment. This study was carried out in accordance with the recommendations of 'Otago Human Participants Ethics Committee' with written informed consent from all participants. All participants gave an informed, written consent to participate in the experiment, in accordance with the Declaration of Helsinki and met the exclusion criteria set for the experiment (non-smoker, in a healthy condition and have NZ-European descent-*appendix 2*). The experiment was approved by Otago Human Participants Ethics Committee (Reference: H16/148) and registered to the Australian New Zealand clinical trials registry (ANZCTR; registration ID: ACTRN12617000034336, Clinical trial name: MODOLF).

2.1.2 Procedure

Each participant was given a brief introduction to all the stages of the experiment and were instructed to attend three, 30 to 45-mins sessions, with a minimum time of these sessions being 24 hrs apart. The experimental room was an isolated environment with air conditioning, allowing for a consistent temperature (23 ± 1°C) in the absence of any olfactory or visual stimuli representing food or distractions. The participants were

instructed not to sniff during the olfactory tests to eliminate the potential effects of sniffing itself during the olfactory tests.

The first experimenter seated the participant directly opposite, on a seat. A consent form and exclusion criteria sheet (*appendix 2*) were signed to qualify the participants for the study. A brief explanation of the full experimental process and the olfactory test were given to the participant. Pre-stimulation (Pre-S) OTT was performed and then the second experimenter conducted the allocated stimulation parameter (high frequency MNS, low frequency MNS or placebo) that was delegated randomly for each participant (enforcing the double-blind design). Lastly, post-stimulation (Post-S) OTT was performed after the allocated stimulation parameter (*Figure 2.1*).

The experimental design of *Experiment 1* is in *Figure 2.1*. A within-participant design was enforced. In each session, participants were randomly assigned to one of the three experimental conditions: high frequency MNS, low frequency MNS or placebo. The order of experimental conditions was counterbalanced across the participants. In addition to the exclusion criteria put in place to ensure that all participants were in healthy condition for the experiment (*see appendix 2*), UPSIT-OIT (Sensonics International, Haddon Heights, NJ 08035 USA) and ODMT (Sensonics International, Haddon Heights, NJ 08035 USA) were performed by each participant, after the placebo session. The results from these olfactory tests were compared to that of previous studies (65,66) to ensure that all participants met a standard criterion of normative, healthy response.

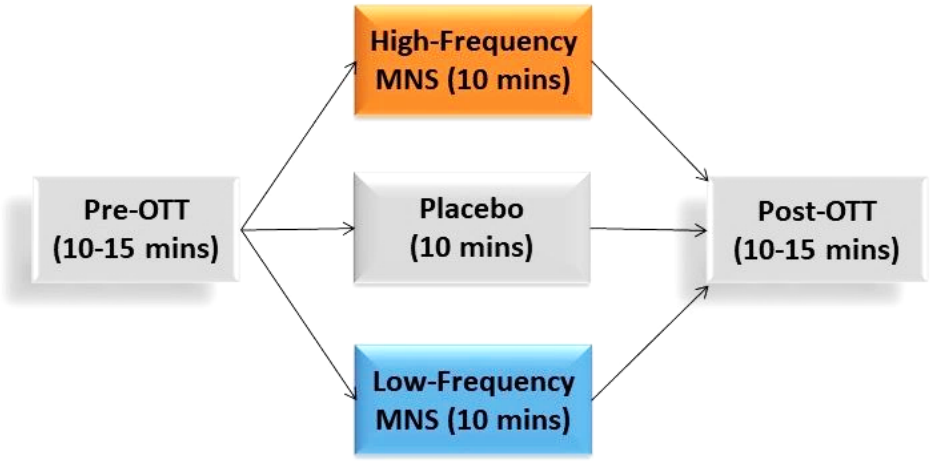

Figure 2.1. Schematic sequence of each stage of the Experiment 1 (non-invasive, MNS-indirect stimulation of the VN).
The sequence of the experimental stages includes pre-stimulation odour threshold test (Pre-OTT), allocated stimulation parameter, post-stimulation odour threshold test (Post-OTT). A brief five-minute introduction, prior to the experiment comprised of obtaining participant's consent and device set up. Labels in diagram: median nerve stimulation (MNS), Odour threshold test (OTT).

2.1.3 Application of median nerve stimulation

In previous animal and human models, MNS was applied through the stimulation of the Neiguan point (PC-6), located in the groove caudal to the flexor carpi radialis and cranial to the superficial digital flexor muscles, roughly 3 cm proximal to the corpus (10,11,13,15,16,142,143,154–156) (*Figures 1.5 and 2.2*). Non-invasive MNS was applied using 'TENS ECO-2' (SCHWA-MEDICO, France) by the same experimenter, using TENS for all three different parameters: high (80 Hz) frequency MNS, low (10 Hz) frequency MNS and placebo (no stimulation but the electrodes were still attached). TENS stimulation in the current experiment was performed with the use of two pairs of disposable rubber electrode-pads, placed on the MN region of the left forearm, with the cathode being 3 cm proximal to the anode (*Figure 2.2*), similar to previous studies using MNS with TENS (158,159). The TENS intensity was adjusted to obtain a tingling sensation over the MN territory of the palm (*Figure 1.*5) and visible flexion response of index and/or middle finger (and/or thumb) so that it was confirmed that there was the

stimulation of the sensory and motor fibres of the MN in each stimulation session. In addition, the stimulation intensity was also kept to a comfortable range for the participant to ensure there was no perception of pain. The strength of the MNS (amplitude) was between 8-15 mA and the pulse bandwidth was 180 μs width bipolar square waveform.

Figure 2.2. Location and set-up of the non-invasive MNS (indirect VNS) (*Experiment 1*).

2.1.4 Data analysis

The data obtained from the OTT was first checked for normal distribution using normality tests with *Shapiro-Wilk correction* (222), performed on the differences between the scores, before and after stimulation for all three parameters (high frequency MNS, low frequency MNS and placebo). Data for participants under high frequency MNS and placebo condition passed the normality test ($p > 0.05$). Thus, a paired sample t-test was performed on these data. The data for low frequency MNS failed the normality test ($p < 0.05$), so a non-parametric [Wilcoxon signed rank test-used to compare two paired samples when the assumptions for the paired t-test are not satisfied) was performed as a more suitable method than transforming the data into a normal distribution, given our sample size (223–225). All the analyses were performed using SPSS software (IBM SPSS Statistics, Ver. 20, St Leonards, NSW).

2.2 Experiment 2: Use of non-invasive, direct auricular vagus nerve stimulation to modulate olfactory functions

2.2.1 Participants

Eighteen Caucasian-male, healthy, non-smokers (age range = 21-38 years, mean: 24.55 years, standard deviation: 3.8 years) participated in *Experiment 2*. Before the experiment, each participant was asked to abstain from food and beverages (aside from water) for 2 hrs preceding the experiment. They were told to abstain from applying any fragrance product/s on the day, prior to the experiment. This study was carried out in accordance with the recommendations of 'Otago Human Participants Ethics Committee' with written informed consent from all participants. All participants gave an informed, written consent to participate in the experiment, in accordance with the Declaration of Helsinki and met the exclusion criteria set for the experiment (non-smoker, in a healthy condition, NZ-European descent-*appendix 2*). The experiment was approved by Otago Human Participants Ethics Committee (Reference: H16/148) and registered to the ANZCTR (registration ID: ACTRN12617000034336, Clinical trial name: MODOLF).

2.2.2 Procedure

All participants were first informed that the attendance of three, 60 mins sessions with a minimum time of these sessions being 24 hrs apart would be required. The experimental room was an isolated environment with air conditioning and had no presence of any olfactory or visual stimuli representing food or distractions. A consistent temperature of 23 ± 1°C was also kept in the experimental room. Each participant was instructed to avoid sniffing during the olfactory tests to avoid its potential effects on olfactory tests or NIRS recordings.

The participant was seated directly opposite the first experimenter. First, a consent form and an exclusion criteria sheet were signed by each participant (*see appendix 2*). This

was followed by a brief explanation of each of the experimental stages, including the use of NIRS and the two different olfactory tests (OTT and STT) that would be performed. NIRS electrodes were placed on the participant, followed by each stage of the experiment (*Figure 2.3*). After the pre-stimulation olfactory tests (Pre-S OTT and Pre-S STT), the second experimenter conducted the allocated stimulation parameter (high frequency VNS, low frequency VNS or placebo) that was delegated randomly for each participant (enforcing the double-blind design). This was followed by the post-stimulation olfactory tests (Post-S OTT and Post-S STT).

Experiment 2 followed a within-participant design. In each session, participants were randomly assigned to one of the three experimental conditions: high frequency VNS, low frequency VNS or placebo. Orders of experimental conditions were counterbalanced across the participants. Supplementary to the exclusion criteria put in place to ensure that all participants were in a healthy condition for the experiment (*see appendix 2*), OIT (Sensonics International, Haddon Heights, NJ 08035 USA) and ODMT (Sensonics International, Haddon Heights, NJ 08035 USA) were completed by each participant, after the placebo session. The results from these olfactory tests were compared to that of past studies (65,66) to ensure that each of the participants met a standard criterion of normative, healthy response.

2.2.3 Application of non-invasive, direct auricular vagus nerve stimulation

Non-invasive, direct auricular VNS was applied using 'TENS ECO-2' (SCHWA-MEDICO, France) by the same experimenter, using TENS of three different parameters: high frequency VNS (80 Hz), low frequency VNS (10 Hz) and placebo condition (no stimulation but the electrodes was still attached). In the current literature on cranial nerves, it has been indicated that the parasympathetic axons of VN influence the human ear with the support of two divisions of the AbVN. The first division of the AbVN follows the external ear canal dispersing primarily to the cavum and cymba conchae area of the auricular skin. The second branch of the AbVN passes through the posterior

auricular nerve of the facial nerve, trailing the route of the posterior auricular nerve (171–173).

To stimulate both division of the AbVN, the current experiment implemented VNS to the internal (covering the cavum concha and extending to cymba concha) and external portions of the left ear (*Figure 2.4 A and 2.4 B*). The left VN is the preferred side of stimulation as it avoids cardiac effects, in comparison to the right VN, which innervates the cardia atria (134). The strength of the VNS (amplitude) was between 10-15 mA and the pulse bandwidth was 180 μs width bipolar square waveform. Stimulation through the AbVN was only performed if there was no perceived pain by the participant and the strength of the VNS (amplitude) was selected to meet a comfortable range for each participant, to ensure stimulation but avoid any perception of pain.

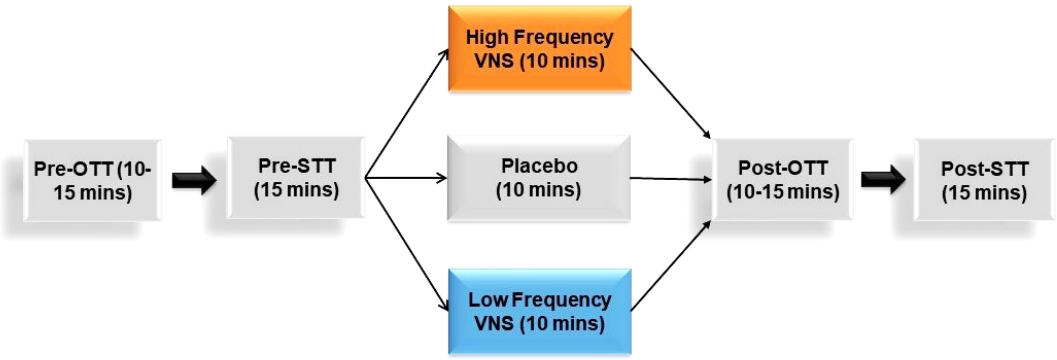

Figure 2.3. Schematic sequence of each stage of the *Experiment 2* (non-invasive, direct auricular VNS).
The sequence of the experimental stages includes pre-stimulation odour threshold test (Pre-OTT), pre-stimulation supra-threshold test (Pre-STT), allocated stimulation parameter, post-stimulation odour threshold test (Post-OTT), post-stimulation supra-threshold test (Post-STT). A brief five-minute introduction, prior to the experiment comprised of obtaining participant's consent and device set up. Labels in diagram: Odour threshold test (OTT), supra-threshold test (STT), vagus nerve stimulation (VNS).

2.2.4 Data analysis

Data from the NIRS was transferred to the 'INVOS' software (INVOS Analytics Tool, Ver. 1.2.1, Minneapolis, USA), which accommodated data presentation. We used average (mean) recordings of venous weighted regional oxygen saturation ($VWrSO_2$ %) of specific time periods marked for each experimental stage (*Figure 2.3*), methods used in the analysis of NIRS in previous research (212,226). Repeated-measures analyses of variance (ANOVA) were performed separately to the data obtained from all three stimulation parameters (high frequency VNS, low frequency VNS and placebo) for assessing $VWrSO_2$ (%) changes in the left and right hemispheric OFC across the corresponding stages of the experiment (Pre-S, stimulation and Post-S) within participants (n=18). Specifically, repeated-measures ANOVA was applied to the OTT and STT periods separately in order to provide synchronised analyses of NIRS data with the functional olfactory tests and stimulation (i.e., Pre-S STT, Stimulation, Post-S STT periods of NIRS and Pre-S OTT, Stimulation, Post-S OTT periods of NIRS).

In addition to the 3-stage analysis of NIRS, an additional 5-stage analysis of NIRS (Pre-S OTT, Pre-S STT, stimulation, Post-S OTT, Post-S STT, in the order of experiment, see *Figure 2.3*) were also performed to reveal any potential influence of the previous olfactory test over the next one that followed correspondingly (OTT effects on STT). Post hoc test using *Bonferroni correction* [for multiple pairwise comparisons between the stages of *Experiment 2* (227,228)*]*, was applied to understand any significance at alpha level of 5% ($p < 0.05$) in all ANOVA tests. To ensure the baseline activity was consistent across three different stimulation parameters for OTT, STT and NIRS data, repeated measures ANOVA was also applied to data that was obtained at the Pre-S stages (*Table 3.3*).

For data obtained from the olfactory tests, normality tests using the *Shapiro-Wilk correction* (222) were performed on the differences between the scores before and after stimulation for all three parameters (high frequency VNS, low frequency VNS and placebo). This test was performed separately for the OTT and STT data. Data for participants under low frequency VNS and placebo condition for OTT and for participants under placebo condition for STT, passed the normality test ($p > 0.05$). Thus,

a paired sample t-test was performed on these data. The rest of the data (high frequency VNS for both OTT and STT tests, and low frequency VNS for STT test) failed the normality test ($p < 0.05$), so a non-parametric (Wilcoxon signed rank test) (223–225) was performed for this set of data. All the analyses were performed using SPSS software (IBM SPSS Statistics, Ver. 20, St Leonards, NSW).

2.3 Information on the functional olfactory tests and near-infrared spectroscopy

2.3.1 Odour threshold test – *Experiment 1 & 2*

The OTT was performed using 'Snap & Sniff Olfactory Test System'-Sniffin' Sticks (Sensonics International, Model: 02400, Hadden Heights, NJ). The Sniffin' Sticks test is a validated psychophysical tool allowing detailed evaluation of a patient's olfactory performance with high levels of sensitivity and specificity (71). This testing battery comprises five blank-odour pens and 15 odorant pens (phenyl ethanol; concentration ranges: 10^{-2} to 10^{-9}, 0.5 log apart). Inter-stimuli-interval was approximately 3 s; the inter-trial-interval was approximately 20 s. The experimental procedure for the OTT followed that of previous studies (68,229). A two-alternative forced choice, two-down, one-up staircase method was implemented to obtain odour threshold measures, following existing literature (68).

In each trial, two pens were presented in a randomised order, one blank and one odorant pen, at 2 cm away from the nostrils. The participant was asked to indicate, which pen contained the odorant. Two consecutive correct identifications led to one-step down in the concentration series for the next trial. One incorrect response incurred an increase in concentration by one-step up. The initial presentation was always at the same concentration step (10^{-6}). As the test progressed, reversal points could occur when concentration of the presented odour changed direction (i.e., increasing to decreasing; or decreasing to increasing). Termination of the test was signalled by completion of seven

reversal points. Individual thresholds were estimated using the standard data analysis method for the staircase test, by averaging the concentrations at, which the last four reversal points occurred (68,229).

2.3.2 Supra-threshold test – *Experiment 2*

STT was performed according to existing literature (81) using the odorant 'Vanillin' (Sensient; CAS number: 121-22-5; purity: 99 %). A series of aqueous solutions with varying concentrations – 8ppm (parts per million), 16ppm and 32ppm – were prepared using a dilution method. The blank samples in the STT consists of distilled water. The STT comprised of three trials, with each trial consisting of five samples in, which two were the target samples and three were the odourless samples. Participants were asked to identify both of the target samples. There was a 30 s delay between each sample (five per trial) and a further 1 min delay between trials (three trials per stage- Pre-S STT and Post-S STT). Participants were awarded a point if both target odours were identified and a maximum of three points could be attained.

2.3.3 University of Pennsylvania Smell Identification Test – Odour Identification Test – *Experiments 1 & 2*

UPSIT-OIT is a standardised test (The Smell Identification Test™, Sensonics, Inc, Haddon Heights, New Jersey), which requires participants to identify, in a four-alternative multiple-choice format, each of 40 odorants presented on micro-encapsulated 'scratch and sniff' labels (65,66). As an example, a test item could read 'This odour smells most like: (a) chocolate; (b) banana; (c) onion; or (d) fruit punch'. The participant must provide a response even if no odour is perceived (the test is forced-choice). The dependent measure is the number of items correctly answered. The odorants are embedded in a 10-50 µm urea-formaldehyde polymer microcapsules fixed in a proprietary binder and positioned on brown strips at the bottom of the test booklets. The stimuli are released by scratching strips with a pencil tip in a standardised manner.

Above each odorant strip is a multiple-choice question with four alternative responses. One point is awarded per correct multi-choice answer, with the total of 40 points that can be attained.

2.3.4 Odour Discrimination/Memory Test – *Experiments 1 & 2*

ODMT is a 12 trial test, using microencapsulated, 'target odorants' where an odour is presented to the participant followed by four odorants from, which the subject is then instructed to select the odour that is identical to the target (first) stimulus. On one-third of the trials, a 10 s interval was present between the presentation of the target stimulus and the first of the four alternatives. The 10 s interval is delayed further to 30 s and 60 s on the other one-third of the trials (in total, four trials with 10 s intervals, four trials with 30 s intervals and four trials with 60 s intervals). The number of trials in, which the target odour was correctly identified behaved as the primary dependent measure. One point is awarded per correct selection of the first stimulus matching the answer stimulus, with a total of 12 points that can be attained (65,66,230).

2.3.5 Near-infrared spectroscopy - *Experiment 2*

NIRS-COVIDIEN INVOS OXIMETER (Model 5100C-PA, Mansfield, MA) was used to record each participant's activity from the left and the right hemispheric OFC in *Experiment 2* (set up as shown in *Figure 2.4 A-B*). NIRS-INVOS uses two near-infrared light sources at two different wavelengths (730 and 810 nm) (199) and two photodiode detectors. The near-infrared light sources travel to either, a proximal or distal detector, in a parabolic path. This enables separate data processing of shallow and deep optical signals. An algorithm is used to subtract the distance of the short, proximal detector from the longer distance of travel from the distal photodiode detector, thus eliminating the inputs of the skin and the scalp (231). This technology through the INVOS-NIRS, allows for the localisation in the area of measurement, while avoiding the contribution of extra-cerebrally reflected photons, and enables high spatial resolution. This technology has been validated in existing literature on human subjects (214,232).

The use of NIRS is possible through the measurement of rSO_2. This is the continuous and non-invasive measure of cerebral regional oxygen balance in the cortex. The 'COVIDIEN INVOS OXIMETER' used in *Experiment 2* is a clinically approved NIRS device, which measures both the venous and the arterial blood in a 3:1 ratio, monitoring brain activity while excluding information from the skin and scalp blood flow. INVOS-NIRS uses a clinically validated algorithm that allows absolute, real-time data accuracy from the cortex (212–214,232–238). With the aid of this algorithm, INVOS measures the $VWrSO_2$ (%) in the cortex, which provides real-time information concerning the balance of oxygen supply and demand and in turn, calculates the venous oxygen reserve (VOR; the remaining oxygen after extraction by tissues and vital organs) (212–214,232–238).

INVOS-NIRS provides several advantages, which eliminates some previous limitations that are presented with the use of NIRS technology. First of all, the wavelength of interest (730-810 nm) has the interference of melanin and water, which are also in the light spectrum of photon absorption. However, the INVOS NIRS have demonstrated in past literature to be unaffected by normal skin pigmentation in adults (239,240). This limitation is also reduced by the use of a within-subject design in *Experiment 2* that reduced potential biasing effects due to inter-individual differences in skin colour. Issues surrounding hair (or hair follicles), which can produce excessive photon scattering in the NIRS, resulting in artefactual low rSO_2 recordings (241) was also eliminated in *Experiment 2* as the recording area was from the forehead that has no hair on the placement of the INVOS-NIRS sensors. The issues surrounding skull and skin perfusion issues, which can also alter the NIRS recordings was addressed through the use of two specific wavelengths of near-infrared light to determine oxygen haemoglobin saturation in the tissue beneath the sensor. With the use of two detectors in shallow and deep locations, this allowed the reduction of interference from superficial tissue (232,242,243).

In addition, INVOS-NIRS allowed the elimination of artefact through motion artefacts of relative movement between an optical fibre and the scalp through the use of signal strength index for each channel by a 5-unit bar scale system. In this system, adequate

signal strength to measure $VWrSO_2$ (%) accurately, is represented through the continuous display of at least one bar (maximum 5 bars) (chapter 6.4.16 and 11.7.4 in the INVOS NIRS 5100c manual (231)). In this context (*Experiment* 2), all sessions were performed at maximum signal (5 bars). Disposable INVOS electrodes were also used for each participant to ensure the highest data quality and keep the experiment hygienic. To ensure maximal stabilisation of the electrodes, cables were held up with a rigid headband (*Figure 2.4A-B*), along with the self-adhesive feature of the disposable INVOS sensors. In the context of the clinical validation and extensive literature of the INVOS-NIRS (over 600 peer-reviewed publications), NIRS-INVOS was used for the present study. With the NIRS, each experimental stage in *Experiment 2* (*Figure 2.3*) was marked and once the data from the NIRS was transferred to 'INVOS' software (INVOS Analytics Tool, Ver. 1.2.1, Minneapolis, USA), it allowed exportation of NIRS data into Excel sheets. Here, an average (mean) recording of $VWrSO_2$ (%) (representing VOR) for the participant from the left and right hemisphere of the OFC for each experimental stage (*Figure 2.3*) was calculated, methods used in previous literature for NIRS data analysis (212,226).

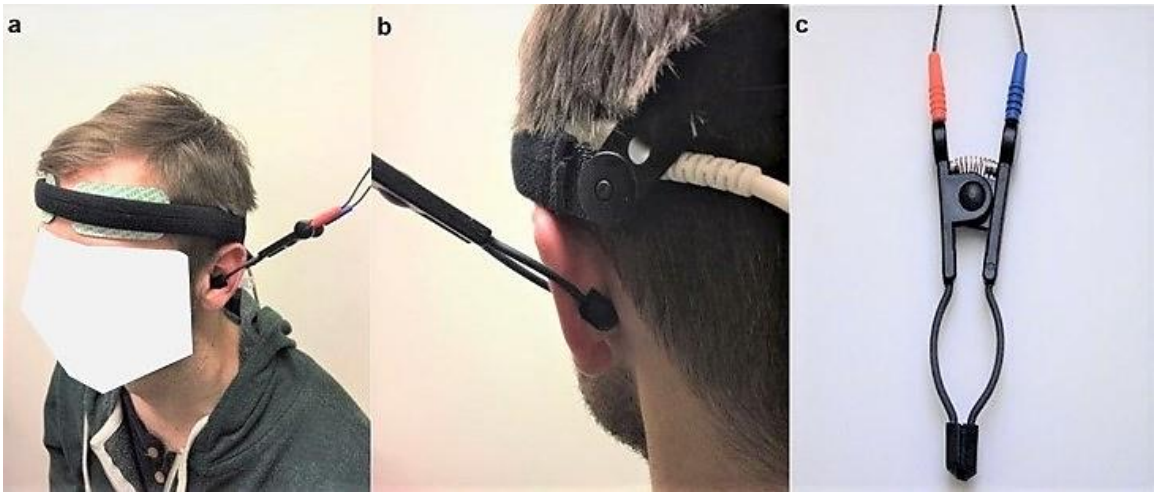

Figure 2.4. Set-up of the NIRS.
(A) Set up of the NIRS electrodes on the forehead for *Experiment 2*. Location and set-up of the non-invasive VNS (Auricular TENS) electrode in the cavum and cymba conchae area of the external ear (*Experiment 2*). **(B)** Location of the non-invasive VNS (auricular TENS) electrode in posterior aspect of the external ear (*Experiment 2*). **(C)** The auricular TENS electrode (*Experiment 2*) (Schwa-Medico, France). Written informed consent was obtained from the participant for the publication of this image (*appendix 1*). Retrieved and edited from Maharjan & colleagues (244).

3.0 Results

3.1 Preliminary pre-screening olfactory test results

The results from the OIT and ODMT is presented in *Table 3.1*. These results were compared to the standard criteria required to meet the normative healthy responses put forward by Doty & colleagues (65,66). Half of the 20 participants displayed values outside of Normosmia in *Experiment 1* while 7 of the 18 participants displayed values outside of Normosmia in *Experiment 2*. However, none of the participants in either of the experiments displayed total anosmia, which was the exclusion criteria for the current experiments and presented olfactory performances in OIT to a standard held in line with the existing olfactory research (65,66). All participants also displayed responses in the ODMT that corresponds to healthy ranges, in line with existing literature (65,66,230,245). In this context, all participants qualified to take part in both of the present experiments.

3.2 Experiment 1: Use of MNS (indirect stimulation of the vagus nerve) to modulate olfactory function

3.2.1 Odour threshold test results

Each participant's (n= 20) results in the OTT, before and after MNS, for all three stimulation parameters (high frequency MNS, low frequency MNS and placebo) are presented in *Figures 3.1 (A-C)*. There were no significant differences in OTT performance after MNS under any of the three stimulation parameters [high frequency MNS, *p*:0.472 (paired sample t-test); low frequency MNS, *p*:0.395 (Wilcoxon signed-rank test); placebo, *p*:0.599 (paired sample t-test)].

Table 3.1 Results from the pre-screening OIT and ODMT from *Experiment 1* and *Experiment 2*.
Participants scores on the OIT and ODMT arranged into the criteria determined by their scores in *Experiment 1* (n = 20) and *Experiment 2* (n = 18). odour identification test range = 0 – 40 (65,66), odour discrimination/memory test range = 0-12 (65,66,245).

Test score	**Odour Identification Test**		**Test score**	**Odour Discrimination/Memory Test**	
	Experiment 1	**Experiment 2**		**Experiment 1**	**Experiment 2**
6-18 (Total Anosmia)	0	0	**0-7**	0	0
19-25 (Severe Anosmia)	0	1	**8**	8	10
26-29 (Moderate Anosmia)	0	0	**9**	1	3
30-33 (Mild Microsmia)	10	6	**10**	1	4
34-40 (Normosmia)	10	11	**11-12**	8	3

3.2.2 Intergroup data from the odour threshold test

The results from the repeated-measures ANOVA to determine if there were any potential differences in the performance of the OTT in the three different stimulation parameters (high frequency MNS, low frequency MNS and placebo) at the Pre-S stage of testing is presented in *Table 3.2*. There were no significant differences in the Pre-S OTT stage for the OTT scores under any stimulation parameter (*Table 3.2*).

Table 3.2. Pre-stimulation OTT result analysis (*Experiment 1*).
Results of the repeated-measures ANOVA that assessed if there were potential differences in the OTT scores of the Pre-S OTT data in all three different stimulation parameters (high frequency MNS, low frequency MNS, placebo) at the Pre-S stage of testing. S.D. = standard deviation, OTT score ranges = 2-9, H = high frequency MNS, L = low frequency MNS, P = placebo condition.

Olfactory Test	Stimulation Parameter	Mean	S.D.	F-Value	P-value
Pre-S OTT	H	5.79	0.85	0.482	0.622
	L	5.79	0.98		
	P	6.00	0.97		

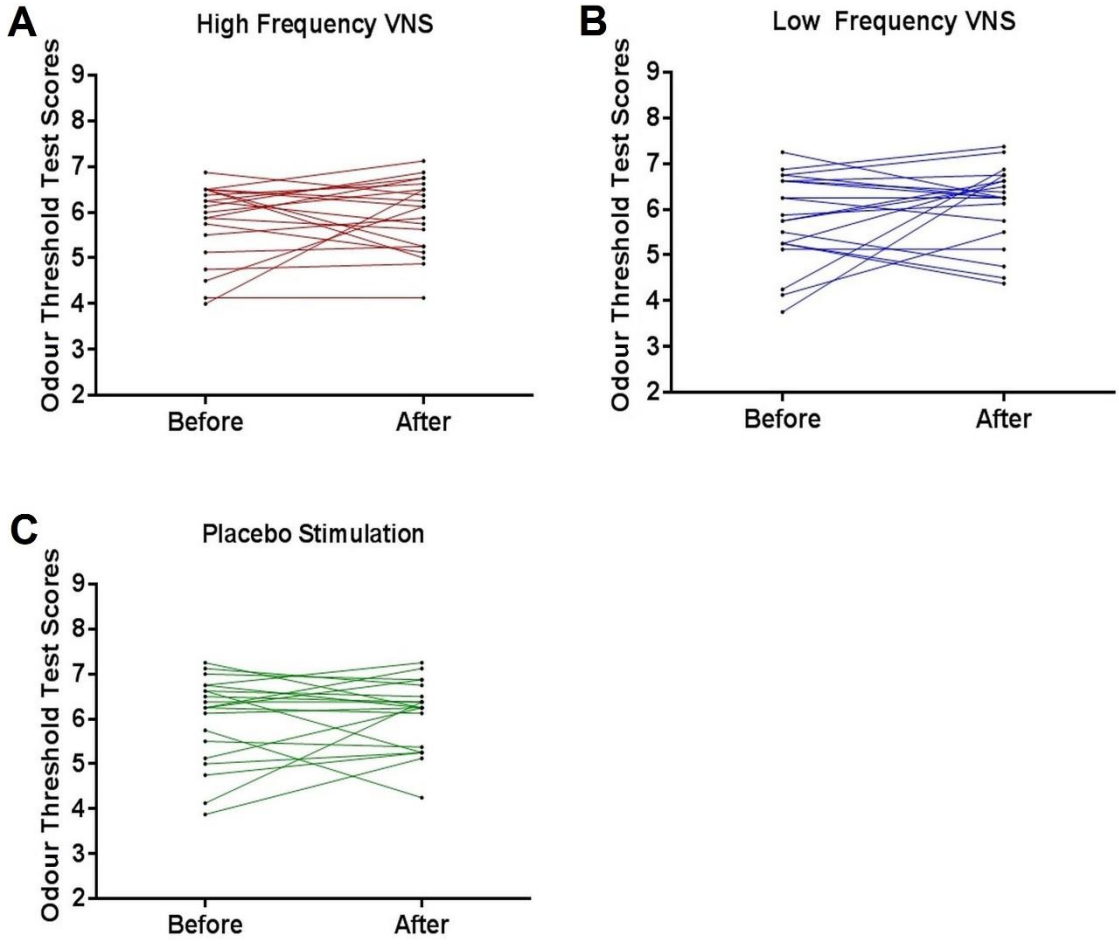

Figure 3.1 A-C. OTT results (*Experiment 1*).
OTT results from each participant before and after each stimulation parameter (high frequency MNS, low frequency MNS and placebo) in *Experiment 1*. Each participant's scores, before (Pre-S OTT) and after (Post-S OTT) each of the stimulation parameters [high frequency MNS (**A**) in red lines, low frequency MNS (**B**) in blue lines and placebo (**C**)] in green lines, for the OTT (OTT scores range = 2 – 9).

3.3 Experiment 2: Use of non-invasive, direct auricular vagus nerve stimulation to modulate olfactory functions

3.3.1 Odour threshold test results

Each participant's results (n = 18) from the OTT, before and after VNS, for all three stimulation parameters (high frequency VNS, low frequency VNS and placebo) is presented in *Figure 3.2*. There was no significant difference in OTT performance after any stimulation parameters [high frequency VNS, *p*:0.523 (Wilcoxon signed-rank test); low frequency VNS, *p*:0.186 (paired sample t-test); placebo stimulation, *p*:0.904 (paired sample t-test)].

3.3.2 3-Stage odour threshold test - near-infrared spectroscopy results (Pre-S OTT, stimulation, Post-S OTT)

In the NIRS recording of the left and right hemispheres in the OFC during the OTT, there were no significant differences in $VWrSO_2$ (%) under all three stimulation parameters in all stages of the experiment (Pre-S OTT, stimulation, Post-S OTT) (left hemisphere, high frequency VNS, *p*:0.643; left hemisphere, low frequency VNS, *p*:0.570; left hemisphere, placebo, *p*:0.061; right hemisphere, high frequency VNS, *p*:0.233; right hemisphere, low frequency VNS, *p*:0.565; right hemisphere, placebo, *p*:0.098). All individual results of 3-stage OTT-NIRS are presented in *Figure 3.2*.

3.3.3 Results of supra-threshold test

Each participant's results (n = 18) from the STT, before and after VNS stimulation, for all stimulation parameters (high frequency VNS, low frequency VNS and placebo) is presented in *Figure 3.3*. Significant differences in STT performances was found after high frequency VNS [*p*:0.021 (Wilcoxon signed-rank test)] but not under the low

frequency VNS [*p*:0.439 (Wilcoxon signed-ranked test) or placebo (*p*:0.083 (paired sample t-test)].

3.3.4 3-Stage supra-threshold test – near-infrared spectroscopy (Pre-S STT, stimulation, Post-S STT)

In the NIRS recording of the OFC in the right hemisphere, there were significant differences in $VWrSO_2$ (%) between the three stages of the experiment after high frequency VNS (*p*:0.031). Post-hoc tests using the *Bonferroni correction* revealed that there were significant differences in NIRS-OFC recording on the right hemisphere between Pre-S STT and stimulation stages (*p*:0.014). There were no significant differences in $VWrSO_2$ (%) between the three stages of the experiment after high frequency VNS in the left hemisphere (*p*:0.253), or after low frequency VNS in both hemispheres (left hemisphere, *p*:0.693; right hemisphere, *p*:0.732) or after placebo (left hemisphere, *p*:0.697; right hemisphere, *p*:0.849). Individual participant results for $VWrSO_2$ (%) from the STT-NIRS stages (Pre-S STT, stimulation, Post-S STT) in both hemispheres of the OFC is presented in *Figure 3.3*. Additional scatterplot chart of each individual participant, before and after stimulation parameters (high frequency VNS, low frequency VNS and placebo), STT scores and the corresponding right hemispheric OFC NIRS recordings (Pre-S STT, stimulation, Post-S STT) are displayed in *Figures 3.4-3.6*.

3.3.5 All stages (5-stage) near-infrared spectroscopy analysis (Pre-S OTT, Pre-S STT, stimulation, Post-S OTT, Post-S STT)

An additional repeated-measures ANOVA analysis of all stages (5-stage) (Pre-S OTT, Pre-S STT, stimulation, Post-S OTT, Post-S STT; *Figure 2.3*) in NIRS, for all stimulation parameters (high frequency VNS, low frequency VNS and placebo) for the left and right hemisphere demonstrated a significance only under high frequency stimulation for the right hemisphere (*p*:0.037) and pairwise comparisons demonstrated

the significance (*p*:0.046, post-hoc tests using the *Bonferroni correction*) only in between the Pre-S STT and stimulation periods of NIRS, similar to the results derived from the 3-stage (Pre-S STT, stimulation, Post-S STT) NIRS analysis.

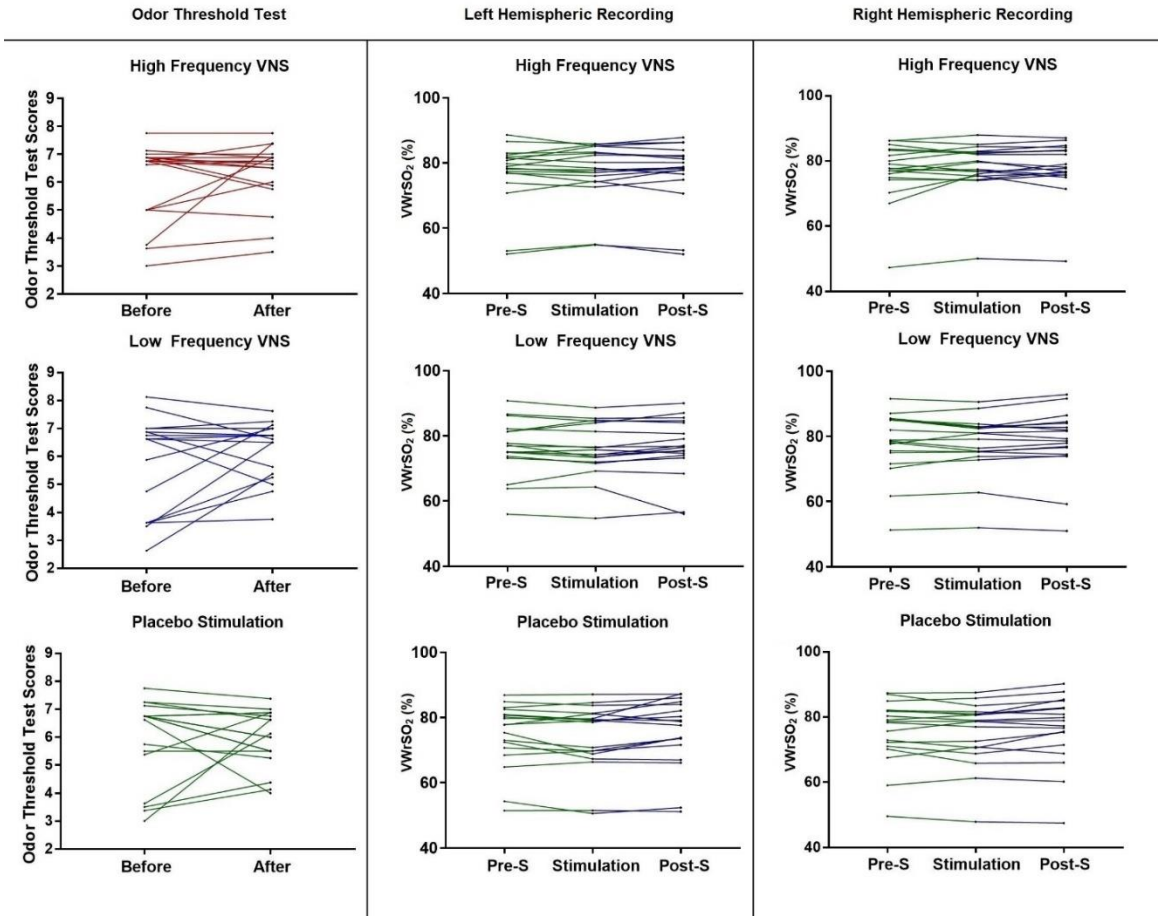

Figure 3.2. OTT and OTT - NIRS recording from the OFC *(Experiment 2*).
Each participant's scores, before and after each of the stimulation parameters (high frequency VNS, low frequency VNS and placebo) for the OTT (OTT scores range = 2 – 9), and each participant's recordings for all three stages of *Experiment 2* (pre-stimulation OTT; Pre-S, stimulation and post-stimulation OTT; Post-S) for all stimulation parameter (high frequency VNS, low frequency VNS and placebo) from the left and the right hemispheres of the OFC, measuring $VWrSO_2$ (%) using NIRS (*Experiment 2*). Retrieved from Maharjan *et al.,* (244).

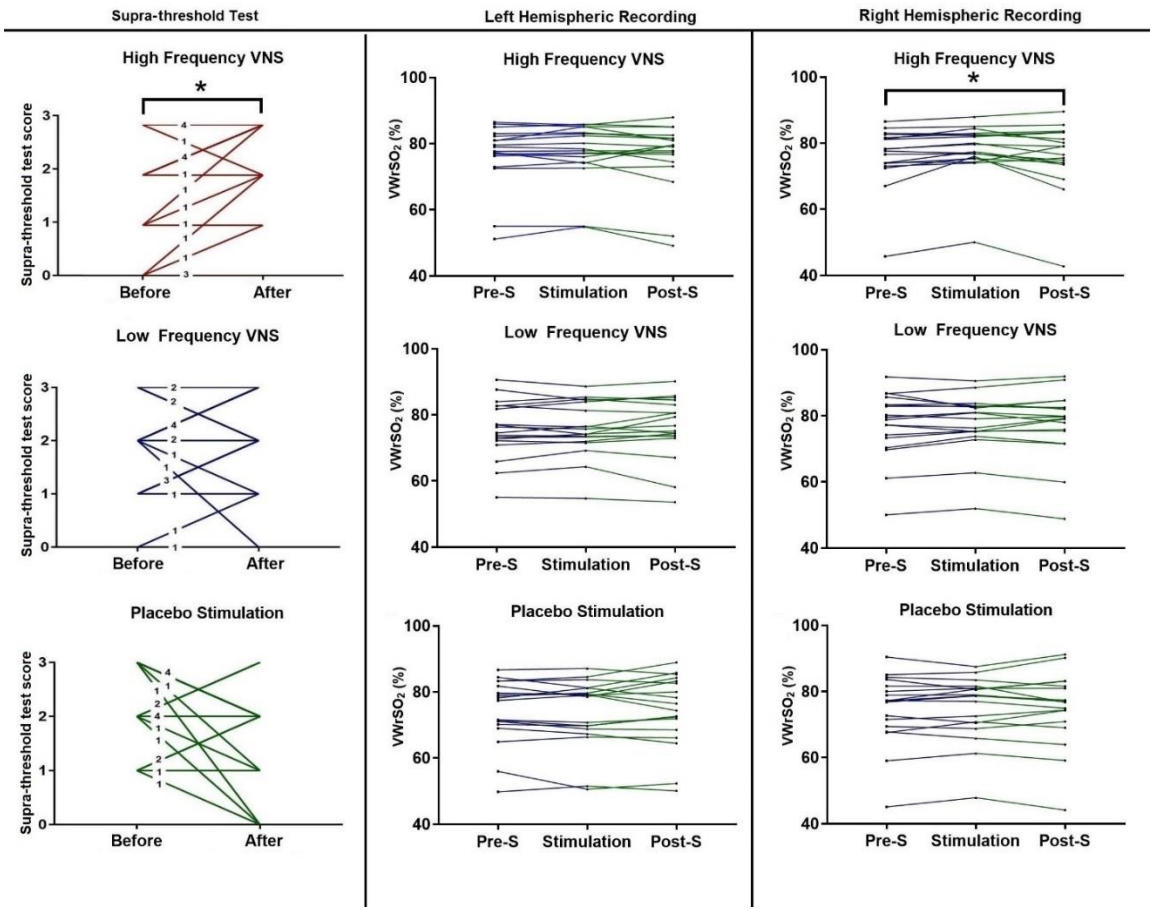

Figure 3.3. STT and STT- NIRS recording from the OFC (*Experiment 2*).
Each participant's scores, before and after each of the stimulation parameters (high frequency VNS, low frequency VNS and placebo) for the STT (STT scores range = 0 – 3), and each participant's recordings for all three stages of the experiment (pre-stimulation STT; Pre-S, stimulation and post-stimulation STT; Post-S) for all stimulation parameter (high frequency VNS, low frequency VNS and placebo) from both the left and the right hemispheres of the OFC, measuring $VWrSO_2$ (%) using NIRS. The numbers in each line for the STT performance represents the number of cases that represent the corresponding result. *Statistically significant ($p < 0.05$). Retrieved from Maharjan *et al.,* (244).

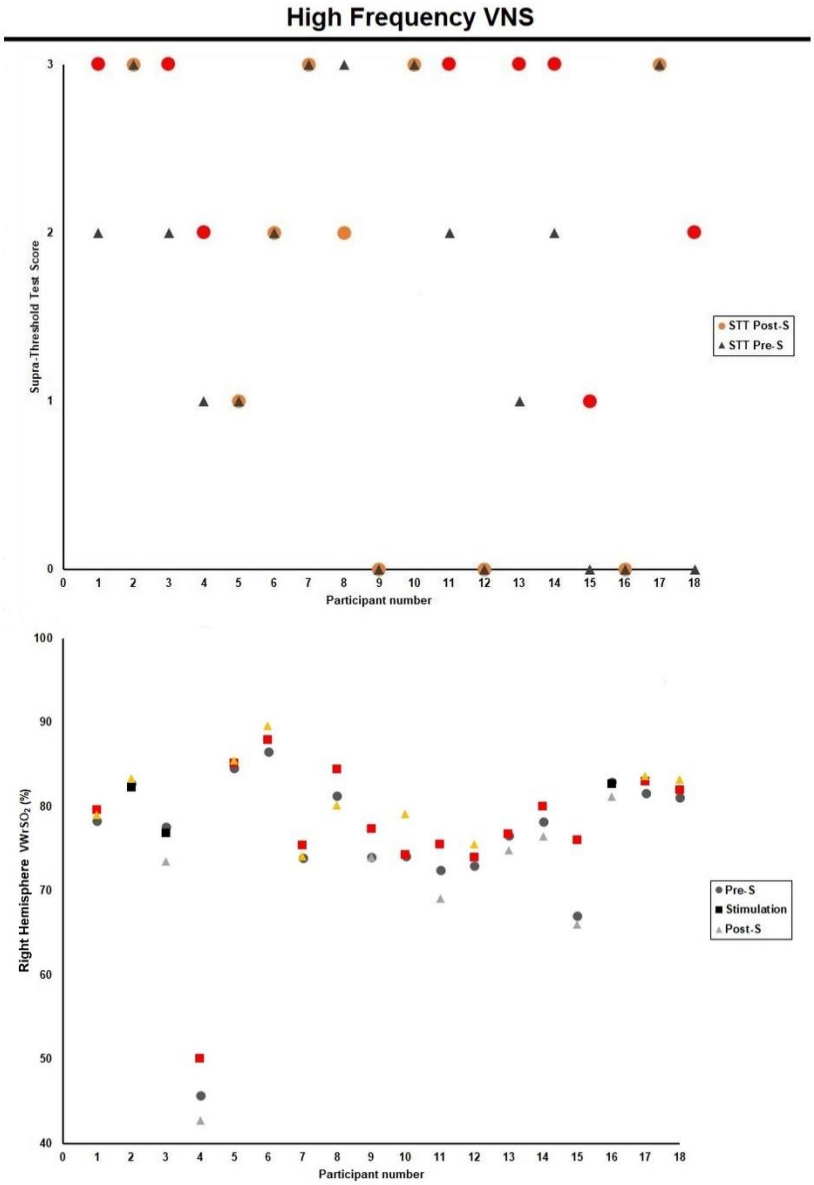

Figure 3.4. Supra-threshold test (STT) and STT - NIRS recording from the right hemisphere of the OFC using direct high frequency VNS (*Experiment 2)*. Scatterplot graph which displays each participant's scores, before and after high frequency VNS for the STT scores (scores range = 0 – 3) in combination with participant's recordings for all three stages of the experiment (Pre-S STT, stimulation and Post-S STT) for the right hemispheric OFC, measuring *$VWrSO_2$* (%) using NIRS. In the STT performance (figure on top), 'red' colour represents participants who improved. On the STT-NIRS of the right hemispheric OFC recordings (figure on bottom), 'Red' colour represents improvements (increased $VWrSO_2$ %) in the stimulation stage from the Pre-S stage and 'Orange' colour represents improvements (increased $VWrSO_2$ %) in the Post-S stage from the Pre-S stage. Retrieved from Maharjan *et al.*, (244).

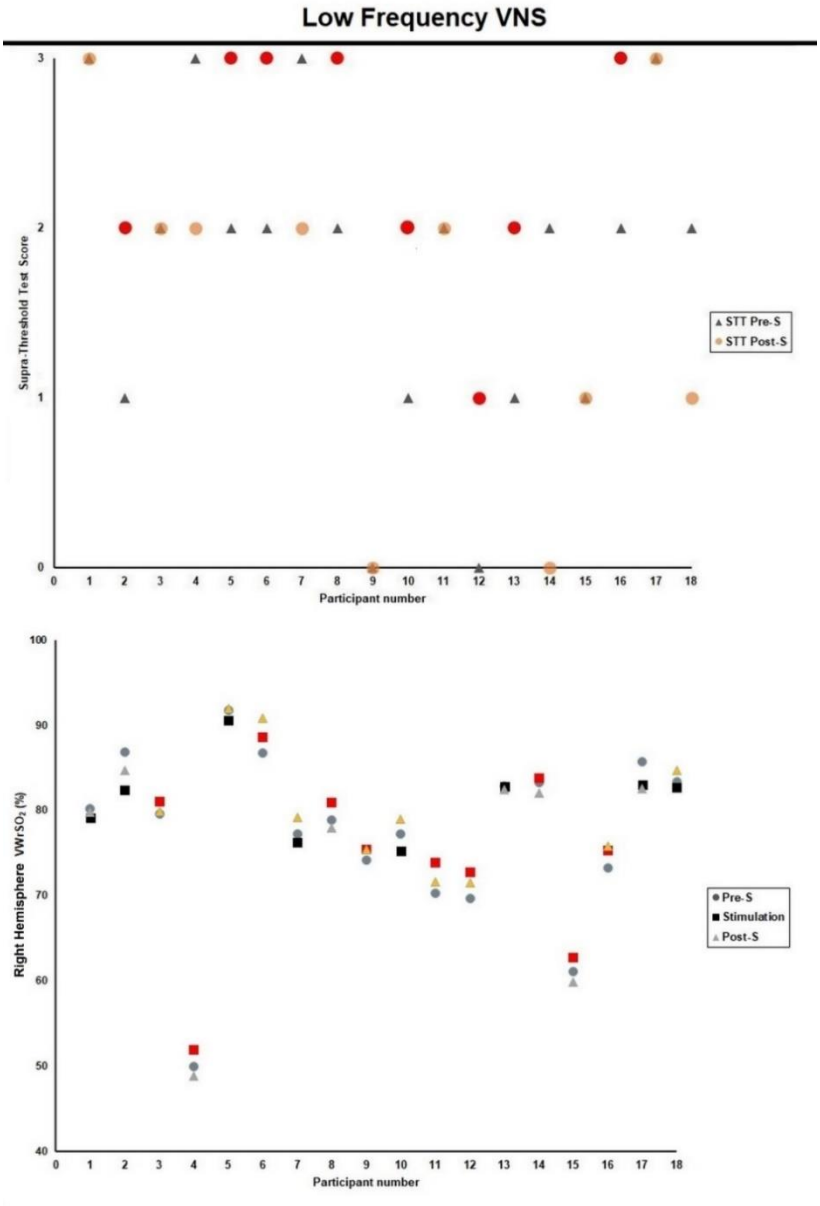

Figure 3.5. Supra-threshold test (STT) and STT - NIRS recording from the right hemisphere of the OFC using direct low frequency VNS (*Experiment 2)*. Scatterplot graph which displays each participant's scores, before and after low frequency VNS for the STT scores (scores range = 0 – 3) in combination with participant's recordings for all three stages of the experiment (Pre-S STT, stimulation and Post-S STT) for the right hemispheric OFC, measuring *VWrSO₂* (%) using NIRS. In the STT scores figure (figure on top), 'red' colour represents participants who improved. On the STT-NIRS of the right hemispheric OFC recordings (figure on bottom), 'Red' colour represents improvements (increased $VWrSO_2$ %) in the stimulation stage from the Pre-S stage and 'Orange' colour represents improvements (increased $VWrSO_2$ %) in the Post-S stage from the Pre-S stage. Retrieved from Maharjan *et al.*, (244).

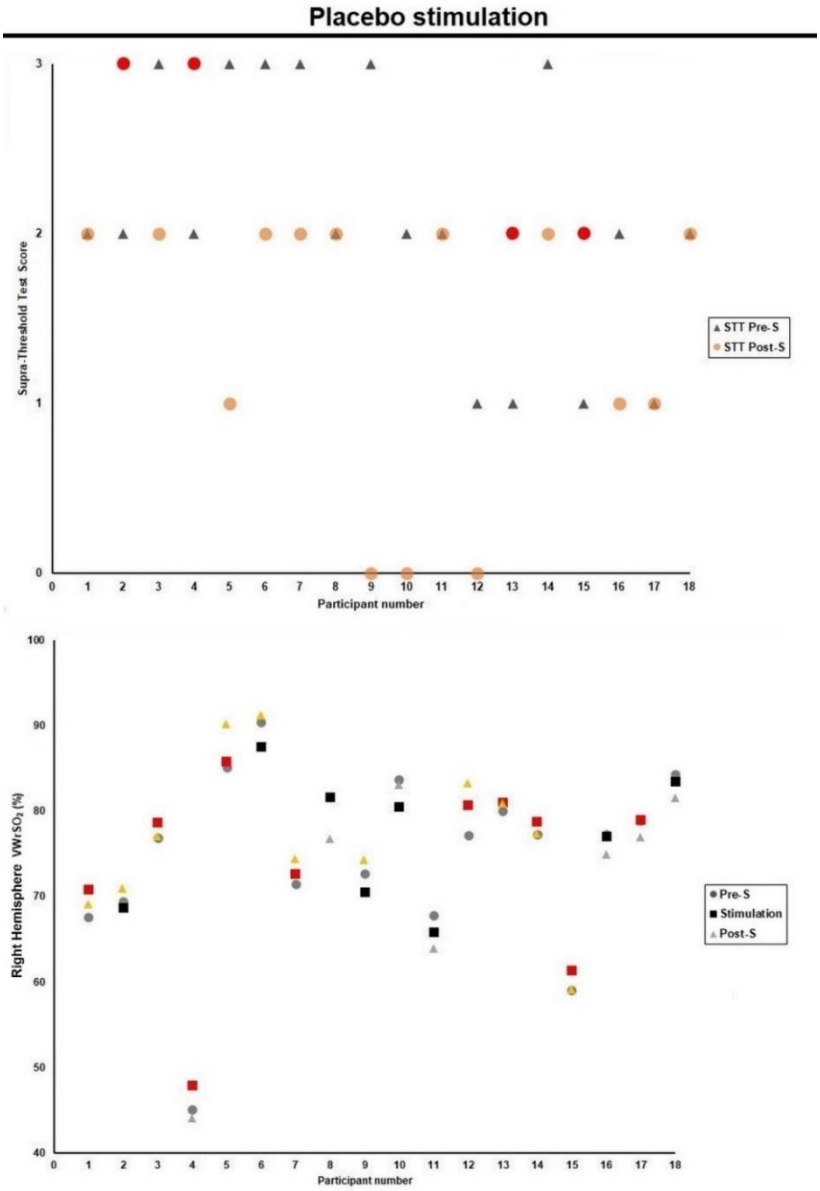

Figure 3.6. Supra-threshold test (STT) and STT - NIRS recording from the right hemisphere of the OFC under placebo condition (*Experiment 2)*.
Scatterplot graph which displays each participant's scores, before and after placebo condition for the STT scores (scores range = 0 – 3) in combination with participant's recordings for all three stages of the experiment (Pre-S STT, stimulation and Post-S STT) for the right hemispheric OFC, measuring *VWrSO₂* (%) using NIRS. In the STT scores figure (figure on top), 'red' colour represents participants who improved. On the STT-NIRS of the right hemispheric OFC recordings (figure on bottom), 'Red' colour represents improvements (increased $VWrSO_2$ %) in the stimulation stage from the Pre-S stage and 'Orange' colour represents improvements (increased $VWrSO_2$ %) in the Post-S stage from the Pre-S stage. Retrieved from Maharjan *et al.*, (244).

3.3.6 Intergroup data from the odour threshold test and supra-threshold test, and respective near-infrared spectroscopy recordings from the orbitofrontal cortex

The results of the repeated-measures ANOVA that examined any potential differences of olfactory tests (OTT and STT) and NIRS analysis ($VWrSO_2$ %) in all stimulation parameters (high frequency VNS, low frequency VNS and placebo) at the Pre-S stage of testing is presented in *Table 3.3*. No significant differences were observed in the Pre-S stage for both olfactory tests (Pre-S OTT and Pre-S STT) or the NIRS analysis periods of both olfactory tests ($VWrSO_2$ %) under any stimulation parameters (*Table 3.3)*.

Table 3.3. Pre-stimulation OTT and STT results and respective OFC-NIRS recordings.
Results of the repeated-measures ANOVA that assessed if there were potential differences in the OTT and STT scores and OFC-NIRS recordings of the Pre-S OTT and Pre-S STT data in both hemispheres in all three different stimulation parameters (high frequency VNS, low frequency VNS, placebo) at the Pre-S stage of testing. *S.D. = standard deviation, OTT score ranges = 2-9, h = hemisphere, H = high frequency VNS, L = low frequency VNS, P = placebo condition*. Retrieved from Maharjan *et al.,* (244).

Olfactory tests and NIRS recordings (H=hemisphere)	Stimulation Parameter	Mean	S.D.	F-Value	P-value
Pre-S OTT	H	6.02	1.41	0.099	0.889
	L	5.83	1.71		
	P	5.92	1.53		
Pre-S STT	H	1.55	1.20	2.006	0.152
	L	1.78	0.94		
	P	2.11	0.76		
Left H Pre-S OTT	H	76.72	9.73	1.156	0.319
	L	76.23	8.48		
	P	74.72	9.84		
Right H Pre-S OTT	H	76.65	9.49	1.663	0.089
	L	75.58	8.85		
	P	74.26	9.91		
Left H Pre-S OTT	H	76.01	9.05	2.129	0.111
	L	78.04	9.81		
	P	75.55	9.74		
Right H Pre-S STT	H	77.35	10.06	2.016	0.149
	L	76.21	9.09		
	P	74.75	10.59		

4.0 Discussion

4.1 Main findings

The overall aim of the current study was to investigate the potential effects of non-invasive, direct and indirect (through the MN) stimulation of the VN on olfactory function. The potential effects of non-invasive, direct and indirect stimulation of the VN was investigated in healthy, adult, male participants using high- and low frequency stimulations. Olfactory sensory tests used in the current experiments included the OTT for non-invasive, indirect stimulation of the VN (using MNS) (*Experiment 1*) and OTT and STT for non-invasive, direct auricular VNS (*Experiment 2*). In addition, NIRS was used to measure $VWrSO_2$ (%) of the OFC during the olfactory tests and neuromodulation of the direct auricular VN (*Experiment 2*).

From the results of *Experiment 1* and *Experiment* 2, the current study has come to two distinct conclusions. First, the use of non-invasive, indirect VNS (through the MN) under high- or low frequencies did not show any modulatory effects on the OTT (*Experiment 1*). And second, to our knowledge, this is the first study to suggest that non-invasive, direct high frequency auricular VNS can positively modulate STT in healthy, adult, male participants (*Experiment 2*). The improvement of the STT performance in *Experiment 2* (non-invasive direct VNS via the AbVN) was supported by NIRS recording of the OFC with increased activation ($VWrSO_2$ %) in the right hemisphere (*Experiment 2*). Post-hoc test (*Bonferroni correction*) after repeated-measures ANOVA revealed that the increase in cortical activation of the right OFC after direct auricular high frequency VNS occurred between the pre-stimulation (Pre-S STT) and stimulation stages (*Experiment 2*).

4.2 Present results in conjunction with existing literature on direct and indirect vagus nerve stimulation

The use of direct (auricular VNS) and indirect VNS (MNS) under low frequencies has been the basis of existing literature in animal models and human subjects. However, no existing literature has focused on the effects of direct or indirect VNS on modulating olfactory function. The use of direct, low frequency VNS in the current literature on human (8,23) have displayed no effect on olfactory performance under invasive and non-invasive techniques. In animal models, direct VNS using low frequency was not able to modulate the activity of the OB (1). The absence of effects in both OTT and STT after direct VNS using low frequency stimulation in *Experiment 2* provides further support to the existing literature (1,8,23).

Use of NIRS (*Experiment 2*) provides additional support, with no significant changes in activation of the OFC in both hemispheres under all three stages of the OTT-NIRS (Pre-S OTT, stimulation, Post-S OTT) or STT-NIRS (Pre-S STT, stimulation, Post-S STT) after direct, low frequency VNS. It is worth mentioning that the exploration of low frequency VNS in the past literature was performed in patients with medically intractable epilepsy (8) and patients with therapy-resistant depression (23). This frequency of stimulation was used as it fit the stimulation parameters for patients with the aforementioned conditions, instead of using stimulation parameters that could potentially impact olfactory function in healthy participants. Due to the conditions of the patients (8,23), it was not feasible for previous research on VNS in humans to test high frequency VNS used in the current study. This is problematic as direct, invasive high frequency VNS (using 80 Hz), was effective in modulating the activity of the OB in existing literature on animal models (1).

In animal models, the use of direct, invasive, low frequency VNS has reported inhibitory effects on the dopamine system that includes the nucleus accumbens, frontal cortex, ventral tegmental area and the striatum (246). In an article by Ziomber & colleagues (246), it was indicated that direct, invasive VNS using low frequencies displayed similar effects on dopaminergic impairments to vagotomy. Dopaminergic system has also been associated with the modulation of ODT and OTT performances

(246). Significant improvements in clinical PD motor symptoms have also been associated with direct, high frequency (130 Hz) auricular VNS (177). In this context, it was hypothesised that although low frequency VNS, through the potential modulation of the dopaminergic system, leads to an impaired VN function, in contrast, high frequency stimulation could potentially have the opposite effects (177,247–249). In addition, other neurotransmission systems that include serotonergic, cholinergic and noradrenergic systems could also be influenced by VNS (*Figure 4.1*), and effects of VNS using separate frequencies (high or low) on these systems needs to be investigated. More research is required to encapsulate the exact mechanism of action of how direct or indirect, non-invasive high or low frequency VNS affects the dopaminergic, serotonergic, cholinergic and noradrenergic systems and in turn, olfactory function.

The use of low frequency MNS in existing literature have been indicated to increase the cortical activation of S-I and S-II (141–144). Previous literature on the MN in both animal models and human subjects used low frequency MNS to improve gastric mobility (12,14–17) and alleviate nausea and vomiting symptoms (10–13,15,16,143). Changes in gastric mobility are driven by the DMV and viscerosensory information of motility and distension is relayed to the NTS and subsequently to the insula-olfactory networks (250) (*Figure 4.2*). Although the use of olfactory functional test (OTT) indicated no improvements after non-invasive low frequency MNS (*Experiment 1*), it is also necessary to test the STT for MNS for a potential change as performed in *Experiment 2*.

In the present study, only non-invasive, direct auricular high frequency VNS demonstrated improvements in STT performance (*Experiment 2).* In addition, there was an increased contralateral activation of the OFC (right hemisphere) under STT-NIRS recordings. The results of direct, high frequency VNS supports existing literature, which suggested that low- and high frequency stimulation could display distinct effects on the autonomic nerve system responses (177,247–249). It should be mentioned that there was a trend to reduce STT functioning in the placebo group (*Experiment 2, Figure 3.3*) but this did not reach significance ($p > 0.05$). This could be associated with sensory adaptation, which can occur rapidly, within a few seconds and recovery from adaptation depends on the initial stimulus. Sensory adaptation can also lead to olfactory fatigue,

which can hinder the participant's ability to detect odours in repeated odours tests (251–253). Future investigations are required to uncover whether 10 mins between pre-stimulation STT and post-stimulation STT is sufficient to recover from sensory adaptation. In addition, intergroup testing of pre-stimulation STT data indicated no significant differences across the three stimulation parameters (*Table 3.3*).

A limitation in the current study (*Experiment 1*) is that the effects of indirect VNS (using high and low frequency MNS) were only observed on lower-order olfactory function (OTT) (74,76). In the exploration of the effects of direct VNS on STT performance (higher-order olfactory function) (74,76), improvements were found in STT performance, in conjunction with an increase in contralateral OFC activation (*Experiment 2*). These results highlight the need to investigate indirect VNS (using MNS) on the higher-order olfactory function (STT). This will help understand whether indirect VNS (using MNS) can exert the same improvements in STT performance to non-invasive, direct auricular VNS. If the same results in STT performance are detected after non-invasive indirect VNS, this would further support existing literature that MNS results in the indirect stimulation of the VN (10,12–17,154,160).

Previous studies have stated that different olfactory measures such as the ODT, OIT or ORT could be processed under the same cortical/subcortical areas associated with olfaction (38). In healthy participants, olfactory functioning in the OTT was highly correlated with that of the OIT (83,254). However, no measures of STT were performed in conjunction with OTT (38). In fact, some articles have suggested that STT measures vary from other forms of olfactory tests (ODT, OMT, OIT), including OTT (82,83). This is supported by our current study (*Experiment 2*) where direct, auricular VNS under high frequency stimulation improved STT performance but it did not affect OTT performance. It is possible that the use of neuromodulation in the current study (*Experiment 2*) facilitates olfactory neurocircuitry responsible for STT but not in neural circuitry responsible for OTT.

It is understood in the present literature that OTT is governed by sections of the cortex that are responsible for low-order olfactory function (such as the OB- peripheral sensory

input of olfactory processes) whereas ODT/OIT/ORT/OMT/STT poses more cognitive load, and are represented in sections of the cortex that are responsible for higher-order olfactory function (27,70,73–76). This could explain the variability seen in *Experiment* 2 where no changes were seen in the OTT after direct, high- or low frequency VNS, or supplementary recordings of the OFC after OTT stages (Pre-S OTT, stimulation, Post-S OTT). It is possible that the lack of improvement seen in the OTT in both experiments of the current study was due to healthy participants presenting OTT scores that equate to the healthy ranges in past literature (65). Investigations into the potential benefits of VN neuromodulation on OTT and STT performance in patients who present olfactory impairments (patients with PD and AD) should be investigated in future experiments.

4.3 Exploration of neuroanatomy underlying direct vagus nerve stimulation

Exploration on the cortical and subcortical structures that are influenced by different frequencies of direct VNS can aid in understanding how VNS can result in the modulation of cortical areas that correspond with olfactory function. In the past literature, only invasive, direct high frequency VNS increased the activity of the OB, with the potential effect of VNS through the periglomerular layer of OB in animal studies (1). It is also understood that the cholinergic elements of the OB begin at the OT (255) and it has been established that the OT modulates the centrifugal control over the OB (*Figure 4.1)* (256). Therefore, this suggests the existence of an oligosynaptic pathway by, which the VN has an effect on the OB through the OT.

The OT is heavily innervated by several neuromodulatory centres in the brain and brainstem. This includes the amygdala, nucleus gemini of hypothalamus, raphe nucleus (RN), locus coeruleus (LC) and the paraventricular thalamic nucleus (NPvt) (257). LC and RN can innervate the OT through noradrenergic and serotonergic fibres respectively (258,259). RN also operates through a dopaminergic pathway to influence the OT, which passes through the ventral tegmental area (260,261). Both the LC (via paragigantocellular nucleus) (262) and RN includes afferents from the NTS and it has

been established that both structures can be stimulated with auricular VNS (137,196,197,263).

The OFC is the secondary order station of the olfactory system that can be modulated by OT or directly by LC/RN (257,262,264–268). LC and RN can influence the OFC directly through the noradrenergic and serotonergic pathways respectively (257,262,268). Supplementary to the OT, the OFC is also susceptible to VNS. VNS could also stimulate the NTS-LC/RN-OFC pathways (137,257,262,263,265,269). Furthermore, LC can also act on the basolateral nucleus of the amygdala and/or paraventricular nucleus (PVN) of the hypothalamus (263,269). Both of these areas acts on the OT (257,269). The NTS can also project directly to the PVN (hypothalamus), which acts on the OT (257,265,268). In summary, it is possible that olfactory structures such as the OT, OB and OFC could be modulated through serotonergic, noradrenergic, cholinergic and dopaminergic pathways through non-invasive, auricular VNS (*Figure 4.1*).

The NTS play an integral role to convey the neuromodulatory effects of the AbVN (137,138). The links between the NTS and OT has been supported by several studies, which observed the responses of OB from the OT when stimulation is provided on the sensory vagus (1,20,21). In summary, due to the numerous interconnections of the OT mentioned in the previous text, it can be considered that OT plays a key role in state-dependent olfactory processing, which can be modulated with auricular VNS (257) (*Figure 4.1).* It is also necessary to mention that separate nerves to the VN (trigeminal and facial nerves) have the potential to modulate the NTS and subsequently, the olfactory system and related olfactory function (270). As detailed in *Figure 1.6B*, in addition to the auricular VN, the facial and trigeminal nerves are also distributed in the stimulation area of the electrodes in *Experiment 2* (271). Therefore, all of these nerves could be stimulated with the electrical stimulation (TENS) in *Experiment 2* and therefore the potential contribution of all of these nerves cannot be excluded to the results of the non-invasive direct auricular VNS.

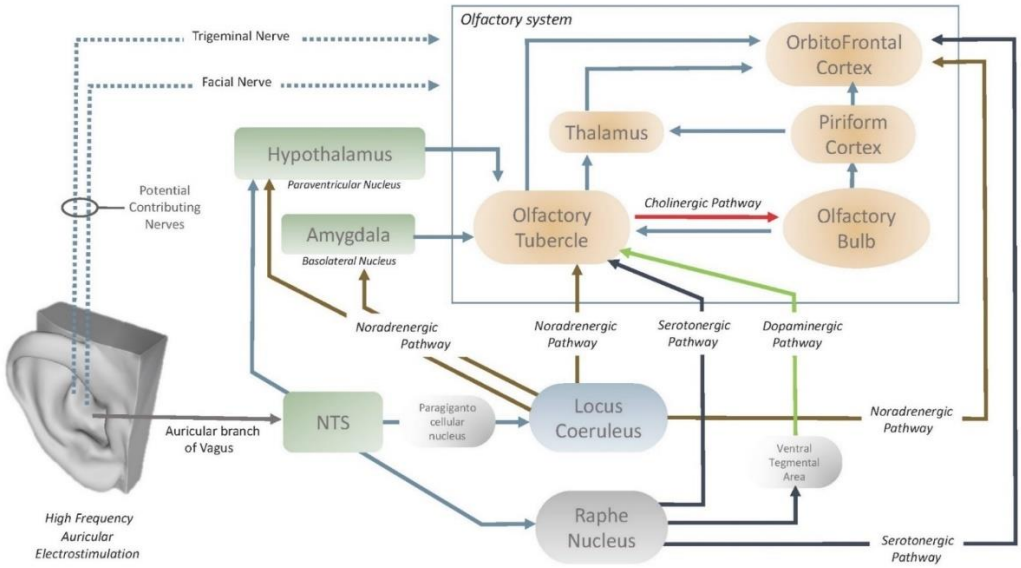

Figure 4.1. The pathway of auricular VN to the olfactory centres.
Coloured arrows indicate the type of pathways in corresponding texts given. Figure is associated with Chapter 4.3 in the current thesis. Label in image: NTS = Nucleus Tractus Solitarius. Retrieved from Maharjan *et al.*, (244).

4.4 Median nerve and its potential connections with the olfactory networks

Exploration of the neurocircuitry in the wake of indirect VNS through non-invasive MNS is central to understanding the potential pathways in, which electrostimulation of the MN could potentially influence olfactory function. In the present literature, three potential pathways have been proposed by, which neuromodulation of the MN could influence olfactory regions in the cortex. This includes indirect stimulation of the VN through the NTS to the olfactory regions, stimulation of the DMV, improving gastric mobility and its subsequent effects on the insula-olfactory regions of the cortex and MNS effects on odour image presentation in the OFC.

In current literature, the NTS is considered a hub of neuroanatomical intersection centre for pathways associated with the peripheral and cranial nerves distributed to the scalp, face, auricular and body (138,196,272). Furthermore, the hub of NTS for the peripheral and cranial nerve stimulation suggests that indirect stimulation of the VN (using MNS)

influences a similar neural network (including NTS) to that of direct VNS (138). MNS reaches the NTS (19,138,156,160,163,168,169) and can influence the OT, OFC and OB via the LC, RN or/and the insula (in the same manner as mentioned in the previous chapter) (*Figure 4.2*).

In addition to the aforementioned neurocircuitry, past literature using fMRI has also indicated that low frequency MNS (in comparison to placebo) can lead to stronger deactivation on hippocampal formation (273). The hippocampal formation is understood to be organised as a unidirectional circuit, made up of the EC, dentate gyrus, CA1 and CA3 subfields and the subiculum (274). The EC is also understood in past literature to serve as a gateway into the hippocampal formation, receiving monosynaptic inputs from numerous regions, particularly the amygdala and olfactory cortices in the context of olfactory processing (274). This is another potential pathway that allows MNS to exert its effects to the olfactory regions, however previous literature has not indicated a direct route from the MN to the EC. One potential route could be through the NTS (VN brainstem nuclei) as previously mentioned, as it is considered the neuroanatomical intersection centre for pathways associated with peripheral nerves at the level of the brainstem (138,196,272).

As mentioned previously, the NTS conveys neuromodulatory effects of the MN through its interactions with the dorsal vagal complex [specifically the DMV (21,138,163)]. The dorsal vagal complex receives a large number of afferent axons from the VN that convey information regarding cardiovascular, respiratory and gastrointestinal sensory systems (138,275,276). This pathway also conveys MNS (indirect VNS) to enhance gastric mobility (12,14,15,17) and reduce symptoms of nausea and vomiting (11,13,16). In a previous study (196), sensitive anterograde tracer was used to trace connections of the NTS within the brain. It was revealed that these sites included the dorsal vagal complex, nucleus gracilis, parasolitary, dorsomotor and hypoglossal nuclei, thalamus (NPvt), hypothalamus and the OT (196). Additionally, all of these aforementioned connections of the NTS were reciprocal (138,275,276).

MNS (indirect VNS) that reaches the DMV is associated with improving gastric functions (11,14,163,277–280,15–19,154,156,160). These vagal afferents from the gastrointestinal tract project back to the NTS (196,250) (*Figure 4.2*), which is integrated in the parabrachial nucleus, where this control of behavioural function is projected to the hypothalamus and amygdala (250). The control of gastric homeostasis of the body is projected to the ventral and dorsal portions of the anterior insula (250) through the thalamus (196). Specifically, the visceral and sensory input is represented in the mid-posterior aspects of the insula while olfactory and gustatory stimuli are represented in the anterior-mid insula, respectively. The projections mentioned above are also supported by intra-operative electrical stimulation studies (250). The insula also receives projections from the primary olfactory regions, OB, OFC, S-I and S-II (250,281), in addition to sharing reciprocal projections with the OFC (29,53) and S-I/S-II (281) (*Figure 4.2*).

Unlike the conscious images that we perceive through the visual system, odour patterns that arise from the OB are thought to present unconscious odour images. These unconscious odour images are associated with pattern recognition due to its complexity and irregularity. Unlike other senses such as vision, perception of smell is heavily influenced by multi-sensory inputs and our ability to detect odours arise from the sub modalities of the somatosensory system (55). Unlike the process of vision, when a smell is perceived in the environment, the spatial image of an odour from the OB is an unconscious one and is thought to reflect the bypass in connection to the thalamus in the human olfactory pathway (55). The MN is involved in a plethora of effects of, which the most prominent being a representor of sensory sensation of touch through the activation of the S-I, S-II and SMA (141–144). It is suggested that when an odour image is perceived through the orthonasal pathway, it travels to the S-I/S-II/SMA, which then connects to the OFC (55). Due to the activation of these somatosensory areas of the cortex (S-I, S-II, SMA), there is potential that MNS could strengthen the odour image presentation to the OFC through the increased activation of the S-I/S-II/SMA. This needs to be investigated using a combination of olfactory functional tests in conjunction with NIRS recordings.

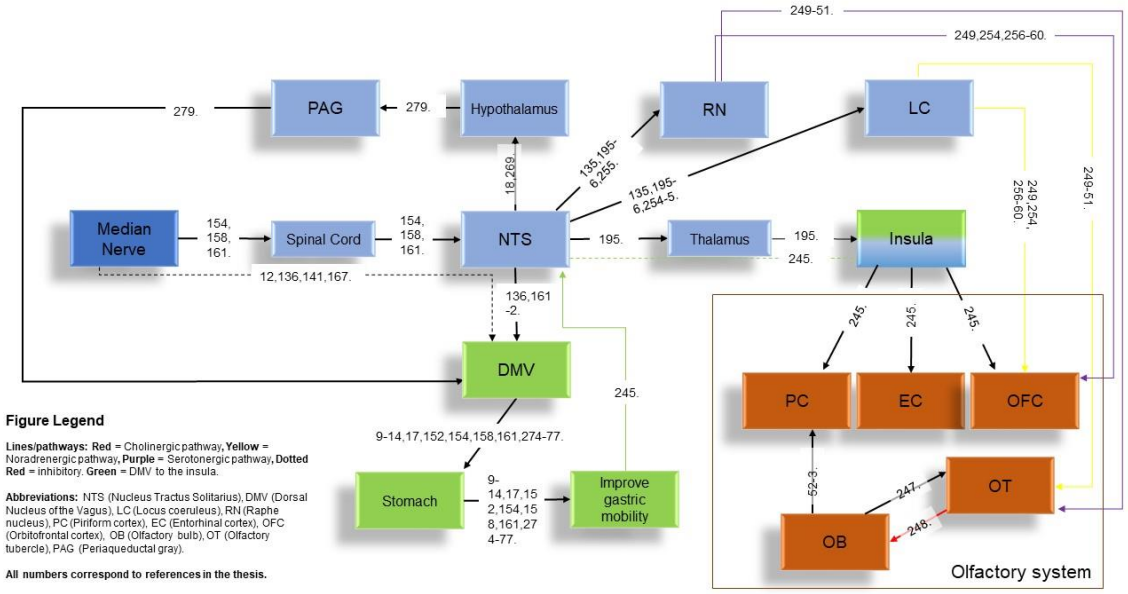

Figure 4.2. The potential pathways to the olfactory network via MNS.
Figure legend in the image indicates the type of pathways (presented by different colours in the image) and all abbreviations used in the image. Structures in colour *blue* refer to the indirect pathway of VNS (median nerve stimulation) to the olfactory network while structures in colour *green* refer to the stimulation of the NTS with the DMV (VN brainstem nucleus) and subsequent interactions with the insula to the olfactory network. *Orange* structures represent the structures of the main olfactory system. Numbers on the pathways indicate references in the current thesis. Abbreviations of structures are given in the Figure Legend on the bottom-left corner of the diagram.

In summary, although there is evidence in the existing literature (the three potential pathways indicated in this chapter) that there are solid neural networks in between the MN and the olfactory system, the present study (*Experiment 1*) could not alter the OTT performance in healthy cohorts. However, potential effects of MNS on STT performance (higher-order olfactory function), in comparison to lower-order olfactory measure of OTT (74,76) in healthy cohorts still need to be investigated in future studies. In addition, potential effects of MNS on OTT and STT performances on patients with neurodegenerative disease with early olfactory impairments also still needed to be investigated.

4.4 Exploration of the primary communication centre of the olfactory cortex (orbitofrontal cortex) using near-infrared spectroscopy during olfactory function

The use of functional imaging techniques, alongside neuromodulation and olfactory testing is a crucial step towards understanding the effects of neuromodulation on the olfactory system. In the current study, NIRS was used to observe the effects of direct auricular VNS on both hemispheres of the OFC (*Experiment 2*). In past literature, studies using PET and fMRI techniques have indicated that passive smelling of odours can activate the amygdala-PC, OFC and the insular-peri-insular cortex (52). Although it is a part of the secondary olfaction regions, the OFC is understood to be the main communication station of olfaction in the cortex (29,30). It is involved in multiple complex sensory pathways that provide input from most of the major sensory and limbic structures, highlighting its role in multisensory integration (27,29,282).

In an article by Savic & colleagues (52) investigations using PET into cortical regions that are activated during different olfactory testing tasks (smelling of passive odourless air, single odours, ODT, odour quality and ORT) were recorded. It was indicated that passive smelling of odours can activate the amygdala-PC, insular cortex, right hemisphere of the OFC, thalamus and cingulate cortex (157,220,221,283). In a recent meta-analysis and review (130), it was reported that patients with PD presented a significantly larger OB volume on the right hemisphere, indicating the presence of lateralised differences between the two hemispheres. In support, several articles have indicated that the right hemisphere is more important for higher-order processing of smell sensation that the left hemisphere (157,284,285). Functional imaging studies attribute the right-side lateralised differences to odours inducing increased activation within the right PC and OFC, which are core anatomical structures in olfactory processing (157). There are also reports that support the notion that right-side dominance is only present when discriminating unfamiliar odours but this effect disappears when the odours are familiar (52). In human studies, use of fMRI studies have also demonstrated that smelling produces an increase in cerebral blood flow at the junction of the inferior frontal-temporal lobes and the right OFC (212,216). Numerous reports using fMRI techniques also indicate a right-hemisphere dominance in OFC activation under odour judgement tasks (50,213,216–220).

Previous studies using functional brain imaging techniques have reported that direct, invasive VNS of 20-30 Hz caused significant increases in blood flow in the bilateral OFC and right EC, which did not occur under 1 Hz. Stimulation of 1-30 Hz however, share similar increased neural activation patterns in the right thalamus, right postcentral gyrus, bilateral (inferior) cerebellum, amygdala, hippocampus and posterior cingulate gyrus (286–289). In comparison to past literature on direct invasive VNS using 20-30 Hz, (8,23,137,286–289) but in support of direct invasive VNS using 1 Hz (286,289), *Experiment 2* in the current study did not display any significant effects under direct non-invasive auricular VNS using 10 Hz in the OFC. In contrast, direct non-invasive auricular VNS under high frequency (80 Hz) displayed significant differences in the contralateral-right hemispheric OFC. In summary, the spectrum of different frequencies of direct VNS results in distinguishable effects on the OFC, with 1-10 Hz exhibiting no effect on either hemisphere of the brain, 20-30 Hz depicting bilateral OFC activation and 80 Hz presenting contralateral OFC activation. Further exploration of the spectrum of different frequencies could be pivotal in understanding why only certain frequencies are able to enhance STT performance (non-invasive, direct auricular VNS of 80 Hz) in comparison to non-invasive, direct auricular VNS using low frequency of 10 Hz.

In comparison to the spectrum of different frequencies using direct VNS explored in the context of cortical activation, the scope of cortical activation using MNS in the present literature has largely focused on S-I and S-II (11,142,144). In these studies, MNS under low frequencies (0.5-2.0 Hz) were indicated to present maximal activation of the S-I and S-II. MNS of 5 Hz resulted in an absence of activation in the S-I, while the use of 15-30 Hz MNS only had effects on some of the participants in the enrolled study (290). The use of MNS in higher frequencies (50 Hz) was also explored in one study and resulted in a reduced activation of the S-I in comparison to the aforementioned lower frequencies of MNS (144). Separate studies also reported that the use of low frequency MNS using 1.5-4 Hz, which displayed increased cortical activation of S-I in comparison to the use MNS of frequencies higher than 4 Hz, in animals models (291) and humans studies (292). Furthermore, previous studies have also reported the use of low frequencies MNS of 10-25 Hz on the modulation of improving gastric function (15,17,19,163). In summary, these reports indicate a lack of exploration of the MN under high frequencies, potentially due to the initial findings of 50 Hz for S-I activation being less effective in cortical activation in comparison to the lower frequencies.

4.5 Clinical implications of VN neuromodulation

Loss of olfactory function is very common in neurodegenerative diseases, which includes AD, PD, vascular dementia, frontotemporal dementia (36), Huntington's Chorea (38,127), alcoholic Korsakoff's syndrome (293,294), Pick's disease (38) and amyotrophic lateral sclerosis (38,295). Olfactory impairment in AD and PD is one of the earliest symptoms and is recognised as a pre-mediator of future pathology in these neurodegenerative diseases. Both of these diseases (AD and PD) present olfactory impairments in over 90% of the patients, with a higher incidence in men (34,36,37). It is also indicated in past literature that olfactory impairments in these conditions could potentially affect separate symptomology of the neurodegenerative diseases. Therefore, this stresses the need to investigate the potential treatment options in olfactory dysfunction in these diseases (36).

Several issues highlight the necessity to investigate olfactory impairments in neurodegenerative diseases. This includes preclinical diagnosis, safety and quality of life concerns (reduced detection of toxic odorants, inadequate nutritional intake and diminished ability to experience pleasure in the context of eating) (38). Other potential issues include the contribution of the olfactory pathways in the pathogenesis of neurodegenerative diseases, link between the pre-symptomatic olfactory disturbance and genetic markers and similarities between neuroanatomical substrates and pathological mechanisms (38). In addition to being an impairment present from the early stages of neurodegenerative diseases such as AD and PD (34,38,41,296), olfactory dysfunction present in neurodegenerative diseases can help differentiate certain conditions. Olfactory impairments can be used to differentiate PD from neurodegenerative conditions that resemble PD such as progressive supranuclear palsy (121,122), 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced parkinsonism (67), multiple system atrophy (122,123), corticobasal degeneration (122) and essential tremor (124,125). Olfactory symptoms also appear alongside non-motor symptoms related to PD pathology, such as autonomic (cardiovascular) and rapid eye movement-sleep behaviours disorders (36,297).

It has also been indicated in the current literature that higher the olfactory dysfunction is at baseline in patients diagnosed with PD, the higher the risk of developing visual hallucinations and greater cognitive dysfunction (34). As indicated in *Figure 1.4* and previous articles by Braak & colleagues (128,133), the initial spread of PD pathology (LN) originates at the DMV due to the vulnerability of long unmyelinated projection cells of this nuclei. In addition, the spread of pathology in the early stages of PD is present in the OB and anterior olfactory nucleus (34,133). This is followed by the presence of PD pathology in the OT, PC, periamygdaloid cortex and EC (133). This supports the presence of olfactory impairments in the earliest stages of PD (preceding the motor symptoms).

A recent article by Doty & colleagues (91) reported that patients with PD had reduced performance in STT in comparison to healthy controls. In *Experiment 2*, healthy participants displayed an improvement in STT performance using direct, auricular high frequency VNS. Significant improvements in clinical PD motor symptoms have also been associated with direct, high frequency (130 Hz) auricular VNS (177). It has been indicated that deficits of olfactory functions are associated with the beginning of symptomology separate to olfaction in neurodegenerative diseases (36). Therefore, this suggests that potential benefits could also extend past solely olfaction if potential amelioration of olfactory impairments were possible.

Comprehension of the mechanisms of plasticity in the olfactory system will enable understanding into brain mechanism for recovery and reorganisational process of the brain. It is also particularly relevant to the loss of olfactory function as one of the initial symptoms in neurodegenerative diseases (such as AD and PD) (41). Therefore, the potential of improving STT performance (in line with increased OFC activation) using non-invasive, direct high frequency VNS on olfactory function in the early stages of PD needs to be investigated further in future studies. This should be performed using the non-invasive approach as it exhibits effects similar to that of invasive approaches (137) but it also eliminates the risk associated with surgical implementation in invasive VNS approaches (192,194). To our knowledge, there has been no report of side effects due to non-invasive, direct or indirect (MNS) VNS in the context of cortical activity and

therefore, a negative effect through the use of non-invasive, direct and indirect (MNS) VNS would be a rare possibility in the context of previous research.

The use of neuromodulation in the current experiments could also be coupled with olfactory training techniques (exposure to training odours twice a day, with exposure involving the use of one deep sniff of the odour) used in past literature (41). In an article by Kollndorfer & colleagues (41), an increase in OTT performance using the same Sniffin' Sticks techniques of the current study was present after olfactory training techniques. In our experiments using non-invasive direct auricular VNS, we found improvements in the STT but not in OTT. There is potential to use both neuromodulation and olfactory training techniques, which could potentially improve OTT and also further improve STT performance. Resolving or potentially improving olfactory functions in both lower-order (OTT) and higher-order (STT) perceptual olfactory processing systems could be important in reducing the overall olfactory impairments present from early stages of AD or PD. Implementation of another duality of treatment methods could include the use of odour deprivation techniques (use of micro foam surgical tape over the nostrils to occlude nasal airflow in an odour-free, negative-pressure room) alongside neuromodulation. Odour deprivation techniques in past literature (298) had an influence on odour quality coding in the OFC. Therefore, if this technique was co-instilled with neuromodulation techniques used in the current study, it would potentially provide supplementary benefits in olfactory test performance to that of VNS.

4.6 Future studies on direct or indirect VN neuromodulation

In the current study, although non-invasive approaches of indirect VNS (using MNS) showed no improvements in OTT performance, direct auricular high frequency VNS exhibited an improvement in STT performance with increased cortical activation of the OFC (right hemisphere). The experiments in the current study have raised several questions that need to be addressed with future investigations in direct and indirect stimulation of the VN. In this chapter, future research ideas are discussed in conjunction

with how these investigations would aid in further understanding of the results from the current thesis.

One major recurring theme from the results of the current study is the need to explore the full spectrum of high and low frequencies using direct (non-invasive auricular VNS) and indirect (non-invasive MNS) VNS. The only effects on olfactory processing (STT performance and OFC activation in the right hemisphere) were present under direct auricular high frequency VNS. As mentioned in the previous chapters of the thesis, although the exploration of stimulations under low frequencies is abundant in both VN (8,23,137,286–289) and MN (17,19,144,163,290–292), there is a lack of exploration of the higher frequencies of both direct and indirect (through MNS) VNS in human studies. Future research using non-invasive VNS under high frequencies of 80 Hz or over should be explored using similar experimental parameters to the current studies. Such exploration has been previously demonstrated for the lower frequencies in both the direct and indirect VNS approaches. In particular, it is indicated that small changes of Hz (1.5-4 Hz in comparison to over 5-25 Hz) under low frequency stimulation of the MN has significant differences in cortical activation of S-I (144,291,292) while use of 1 Hz in comparison to 10 Hz in VNS had different regions of activation in the cortex (8,286–288). These previous studies indicate the importance of exploring small changes in frequencies of stimulation in conjunction to observing the full spectrum of high frequency stimulation using direct and indirect VNS, to encapsulate the range of effects in cortical activation, previously explored for direct and indirect VNS under low frequencies.

To date, there are no studies that have explored the complete pathways from the direct or indirect VNS to the cortical representors of olfactory functions. This indicates a lack of knowledge that should be addressed in future research as more than three decades ago, past research in animal studies have alluded to the effects of direct VNS in modulating the activity of the OB (1,20). Particularly on indirect VNS (MNS), several studies in existing literature have attempted to propose potential pathways from MNS (indirect VNS) to the VN brainstem nuclei (NTS) (*Figure 4.2*). In particular, Songzi & colleagues (156) have previously stated that the MN could exert its effects to the NTS through the spinal cord (C3-T3 section), while another article by Imai & colleagues

(163) suggested that spinal nerves can contribute to gastric function through the NTS (VN brainstem nuclei). However, these studies do not indicate any effects of the indirect VNS to the areas of cortex responsible for olfactory functions. Therefore, future research is required, particularly using fMRI techniques, which would allow the complete exploration of the cortex under neuromodulation (direct and indirect approaches) of VNS. These explorations would legitimise the full neurocircuitry of the direct and indirect VNS to all potential connections in the olfactory regions of the cortex, and also provide support to the neurocircuits that were presented for both direct (*Figure 4.1*) and indirect (*Figure 4.2*) VNS in the current studies.

One future study that should also be addressed is to observe the effects of non-invasive, indirect VNS (using MNS) on STT performance. As non-invasive, direct auricular VNS exhibited improvements in STT performance in the current study, these effects could be replicated with indirect VNS (using MNS). This would further support existing literature that the MN is an indirect stimulation of the VN (10–15,17). Separate olfactory testing modalities to OTT and STT that includes ODT, OIT, OMT and ORT still need to be investigated under direct and indirect VNS under high and low frequencies. This would help answer questions as to whether neuromodulation of these nerves is effective for the full array of olfactory functions. This would also aid in composing a comprehensive map of the cortex that could detail, which parts of the cortex are activated by certain neuromodulatory techniques (direct or indirect VNS). It could also aid in understanding which combination of cortical structures is responsible for the processing of each olfactory testing modality. A particular neuroimaging technique such as a fMRI would allow the observation of the whole cortex in combination with neuromodulatory techniques. A previous fMRI study with low frequency, direct auricular VNS has already demonstrated alterations of numerous different brain regions (137). Therefore, using fMRI to observe primary and secondary olfactory regions in conjunction with olfactory testing and neuromodulation (direct and indirect VNS) is the next step in future investigations.

In existing literature, direct VNS is currently available as a potential therapy for intractable/medically refractory epilepsy (9,138,184,185), chronic treatment-resistant depression (186), pain (186,187), primary headaches and medication-overuse headaches

(188). Despite the fact that the first presence of PD-pathology (LN) is present in the DMV (128) and olfactory impairments precede motor symptoms in PD (34), there is no existing literature on the use of direct VNS as potential treatment for olfactory impairments in humans. Our current study (*Experiment 2*) was the first to indicate that neuromodulation of non-invasive, direct auricular high frequency VNS can improve STT performance (with supplementary increase in cortical activation on the OFC-right hemisphere). This therefore, indicated the need for future research to investigate the potential of direct VNS in improving other olfactory tests that are also impaired in patients with PD (ODT/OIT/ORT) (69,73,106,124,126,127). Furthermore, this study only investigated the use of acute VNS. Investigations into the use of chronic VNS under the same stimulation parameters (high and low frequencies) to the current study in the performance of olfactory tests should also be explored.

Although standard methods were used to measure OTT and STT in the current study, there are numerous approaches in olfactory behavioural tasks that are available to test these functions. Replicating the current experiments using different methodologies to test OTT and STT would enable more validity in the results of the current study and should be explored in future investigations. In addition, the exploration of olfactory function was performed in healthy participants in the current study. As these participants presented healthy responses in ODMT, OIT and OTT (65), future studies should observe the effects of nerve neuromodulation on participants who have impairments in olfactory function prior to the stimulation. Population groups that could be used in future studies that replicate the current study could include patients with AD or PD as they present olfactory impairments from the early stages of respective conditions (34,36,38).

In *Experiment 2*, there were eighteen participants in a within-subject design under three stimulation parameters (high frequency VNS, low frequency VNS and placebo). In addition to presented *p-values* for the statistical significance, the effect size [correlation coefficient (r): 0.39 for the STT with the significant output after direct auricular VNS under high frequency stimulation] revealed a medium/moderate effect [in line with Cohen's thresholds for deducing the effect size (r: 0.1 small effect, r: 0.3 moderate effect, r: 0.5 large effect)] (299). Furthermore, it is understood that the use of 10 mins of

nerve stimulation produces neuromodulatory effects for about an hour after the period of neurostimulation (177). However, the exact time that this effect begins to occur is currently unknown. If the exact time for the onset of the effect was clarified then performing the same experiments during the stimulation period, rather than just after the stimulation, could potentially result with a higher effect-size.

It is also necessary to mention that the present experiments only included healthy, male participants of European-New Zealand descent. Only one ethnicity was used as there are reports of cross-cultural differences in odour perception and rating, independent of sex (300–302). The difficulty in enrolling female participants for the current study was due to the required calibration of the menstrual cycle across the female participants. Future studies that could examine the responses of neuromodulation on separate cohort groups and on female cohorts in the context of hormonal influences over the olfactory system would aid in the exploration of all cohorts in the population in future studies using the similar experimental design to the current study.

4.7 Conclusion

In conclusion, the current study exhibited, for the first time in human research that non-invasive, direct auricular high frequency VNS is effective in improving the healthy, adult, male olfactory function in the STT, accompanied by increased activation of the right hemispheric OFC. In comparison, non-invasive, indirect VNS (using MNS) did not improve the performance of OTT for the healthy, adult, male participants. As olfactory dysfunction is an early and crucial symptom present in neurological diseases such as AD and PD and also in the context of STT impairment in PD, a potential chance to ameliorate such deficits could be essential in early treatment processes. Future research needs to incorporate, as this current study has, direct and indirect, non-invasive VNS with functional imaging of all relevant olfactory regions in the cortex, to gain a complete representation of the cortical structures influenced by neuromodulation of direct and indirect VNS observed in the current study.

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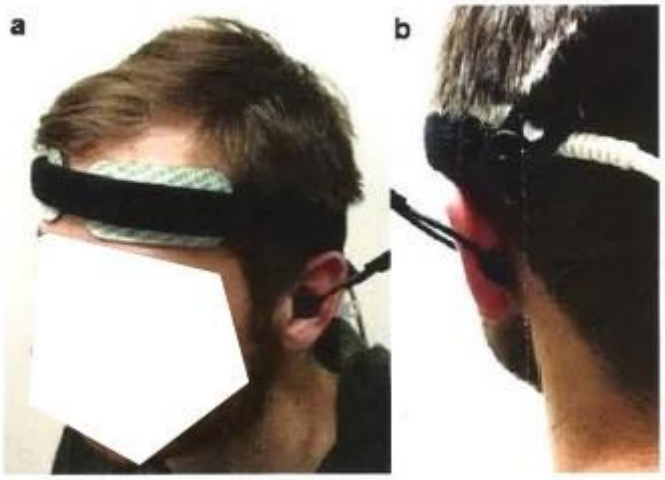
Appendix 1

Participant consent form for Figure 2.4 A and 2.4 B:

To whom it may concern,



I Robert Matthew Graham give permission to the University of Otago, Department of Anatomy to use the photos above, which has full display of my face and body, to be used for scientific purposes. The use of these images will not, in any way, violate the Otago Human Participants Ethics Committee (Reference: H16/148).

Signed _____

Date 23 / 11 / 2017

Appendix 2

Inclusion to study Screening form:

Participant ID:

Age:

Please tick in the boxes below to show that you are eligible to participate in the study:

☐ Are you a male?

☐ Is your ethinicity NZ European (Kiwi-Pakeha)?

☐ Are all four of your grandparents are NZ Europeans as well?

☐ Are you in good health? (no signs of flu, fever, diarrheoa or any sort of illness)

☐ Are you a non-smoker?

Are you on any medication?

☐ Yes ☐ No

Do you have any allergies?

☐ Yes ☐ No

Do you have any sorts of disorders or diseases?

☐ Yes ☐ No

Have you had any of disorders or diseases before?

☐ Yes ☐ No

Candidate's output:

Bates, V., Maharjan, A., Millar, J., Bilkey, D. K., & Ward, R. D. (2018). Spared motivational modulation of cognitive effort in a maternal immune activation model of schizophrenia risk. *Behavioral neuroscience*, *132*(1), 66-74.

Maharjan, A., Wang, E., Peng, M., & Cakmak, Y. O. (2018). Improvement of Olfactory Function With High Frequency Non-invasive Auricular Electrostimulation in Healthy Humans. *Frontiers in neuroscience*, *12*, 1-14.

Maharjan, A., Peng, M., & Cakmak, Y. O. (Submitted for publication). Non-invasive high frequency median nerve stimulation effectively suppresses olfactory intensity perception in healthy males.